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DEAN F. MARTIN, *Editor* BARBARA B. MARTIN, *Co-Editor*

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Biological Sciences

THE VEILED CHAMELEON, *CHAMAELEO CALYPTRATUS*: A NEW EXOTIC LIZARD SPECIES IN FLORIDA

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ABSTRACT: *During field surveys from June 2002 through August 2003, we documented an established population of the veiled or Yemen chameleon (*Chamaeleo calyptratus*) in Fort Myers, Lee County, Florida. We recorded at least 70 individuals, including both genders and all size classes in consecutive years, indicating a reproducing population. Additionally,* ca. *100 individuals were reportedly removed from this site prior to our study.* Chamaeleo calyptratus *has also been reported from areas near Lehigh Acres and Alva, Lee County, and Naples, Collier County, suggesting independent introductions of this popular exotic lizard. Monitoring of this population should continue, and eradication should be attempted if ecological impacts on native species are observed.*

Key Words: *Chamaeleo calyptratus*, veiled, Yemen, chameleon, lizard, introduced, exotic, reptile, Fort Myers, Florida

FLORIDA presently has the largest number of non-native amphibian and reptile species in the United States (Butterfield et al., 1997). Miami is one of the largest ports of entry into the U.S.A. for wild pets, and feral populations of many of these species are now established in the state. Diverse habitats and warm climate have facilitated the establishment and range expansion of exotic species in Florida (Krysko et al., 2003). While conducting recent field studies in southern Florida, we found a new established exotic lizard species, the veiled or Yemen chameleon, *Chamaeleo calyptratus* Duméril and Bibron 1851, in Fort Myers, Lee County. In this paper, we document life history, mode of introduction, and population age structure of *C. calyptratus* at our study site.

Chamaeleo calyptratus is an arboreal lizard species that ranges from Asir Province, southwestern Saudi Arabia, to Aden, Yemen, where it lives on high, dry plateaus up to 2800 m and in foothills, forests, low-elevation maize fields, and inland river valleys (Fritz and Schütte, 1987; Meerman and Boomsma, 1987; Zari, 1993; Showler, 1995; Schmidt, 2001). *Chamaeleo calyptratus* is a habitat generalist; during the daytime, it is mostly found in vegetation 0.2–3 m above ground, although it can be found sleeping higher up in branches (Schmidt, 2001).

Males can reach 62 cm total length (TL) (20–30 cm snout-vent length [SVL]) and females 45 cm TL (10–20 cm SVL) (Schmidt, 2001). Captive specimens can live up to 10 years, with males living a mean of five years and females three years (Schmidt, 2001). Sexual dimorphism is apparent; males possess a tarsal spur on the hind foot throughout life, as well as a hemipenal bulge at the base of the tail and up to 80 mm higher cephalic casque than females as adults (Schmidt, 2001). *Chamaeleo calyptratus* exhibits visual signals for communication, including deliberate body movements, head jerking, and color pattern changes (Barnett et al., 1999; Kelso and Verrell, 2002). Mature males typically have bold vertical body bands of bright yellow, green, and blue mixed with yellow, orange, or black. Mature females are normally light green with shades of tan, orange, white, and yellow. *Chamaeleo calyptratus* does not adapt its coloration to its surroundings; instead, color change is more a physiological response to emotion (Schmidt, 2001) and light. *Chamaeleo calyptratus* is usually a shy, solitary species, but males are very territorial and will combat rival males (Schmidt, 2001). *Chamaeleo calyptratus* produces a low-frequency buzzing vibration that may serve as vegetation-transmitted vibratory signals for communication (Barnett et al., 1999; Schmidt, 2001; Kelso and Verrell, 2002).

METHODS—Records of *Chamaeleo calyptratus* are based on captures and observations during nine survey nights from June 2002 through August 2003 in a vacant, wooded lot *ca.* 1.1 ha in size in Fort Myers (26°40′59.5″N, 81°48′4.5″W). Trees present include laurel oak (*Quercus hemisphaerica*), cabbage palm (*Sabal palmetto*), Indian laurel (*Ficus microcarpa*), and woman's tongue (*Albizia lebbeck*). During the daytime, this diurnal species can be extremely difficult to detect among vegetation. At night, however, adults remain vividly colored while sleeping and perched above ground on tree branches and other vegetation. Chameleons are easy to detect at night using flashlights and headlamps, because light reflects off their scales and causes them to shine (Love, 2002). Captures were made by hand, and voucher specimens and photographs were deposited in the Florida Museum of Natural History (FLMNH), University of Florida (UF collection). Individuals that could not be collected because of dense vegetation or extreme height above the ground were photographed, their TL estimated, and location noted for identification purposes. Only individuals that could be distinguished from others were counted in our overall total. We assigned individuals to one of two age classes based on estimated TL: juveniles < 20 cm and adults > 20 cm TL.

RESULTS—We recorded at least 70 *Chamaeleo calyptratus*, including both genders and all size classes (Table 1; UF 133251, 133255–57, 133259–63, 137030–33). On 25 June 2002, juveniles consisted of two distinct size classes: neonates < 80 mm TL ($n = 8$) and *ca.* 185 mm TL animals ($n = 2$) estimated to be 1.5–2 months old. Neonates were found on blades of grass *ca.* 60–122 cm above ground. Larger individuals were found higher above ground in trees and muscadine grape vines (*Vitis rotundifolia*). Only four of 10 individuals were removed on this first night, including

TABLE 1. Size classes (adults are > 20 cm TL) of the veiled chameleon (*Chamaeleo calyptratus*) recorded in Fort Myers, Lee County, Florida.

Date	N	Juvenile	Adult ♀	Adult ♂
25 Jun 2002	14	10	2	2
28 Jun 2002	15	5	5	5
15 Aug 2002	16	14	2	0
8 Sep 2002	7	4	2	1
5 Nov 2002	6	0	3	3
8 Nov 2002	1	0	1	0
3 Jun 2003	4	2	1	1
18 Aug 2003	4	4	0	0
22 Aug 2003	3	3	0	0
Total	70	42	16	12

one juvenile of each age class and an adult male and female. All individuals were removed on subsequent nights. On 15 August 2002, a neonate was found across the street, and this was the last time a neonate was found in 2002. On 3 June 2003, two neonates and a possible spent female were collected. In August 2003, seven neonates were collected.

DISCUSSION—A reptile dealer in Fort Myers housed *Chamaeleo calyptratus* in outdoor cages at his facility since 2000. These cages were broken into several times by persons intent on stealing animals, and an undetermined number of *C. calyptratus* escaped when cage doors were subsequently left open. In 2001, *ca.* 100 juvenile and adult *C. calyptratus* were collected by the reptile dealer in an adjacent undeveloped lot, indicating that reproduction had occurred at least once in the wild. We believe that neonates found within days of each other were likely from the same clutch of eggs. Therefore, our data suggest that reproduction in the wild has occurred at least seven additional times since 2001, as we found juveniles of two different size classes during each of four surveys in June, August, or September 2002 and neonates during each of three surveys in June or August 2003 (Table 1). Collectors are aware of this site and have removed an unknown number of *C. calyptratus*. We also have reports of *C. calyptratus* from areas near Lehigh Acres and Alva, Lee County, suggesting that this popular pet trade species has been introduced independently elsewhere. On 13 September 2002, an adult *C. calyptratus* (photographic voucher, UF 140472) was collected crossing a road in Naples, Collier County, Florida (Lotz, 2003).

Chamaeleo calyptratus is an extremely prolific species. Sexual maturity can be attained in as little as four months (Schmidt, 2001). In dry habitats in its native range, breeding usually takes place September–October (Schmidt, 2001). Oviposition occurs a few weeks after copulation (Schmidt, 2001). Although *C. calyptratus* has been reported to reproduce once each year, gravid females have been observed throughout the year in some regions (Necas, 1999). In captivity, this species can breed and produce viable clutches of eggs several times each year (Schmidt, 2001). Captive females can oviposit clutches of 12–85 (usually 30–40) soft-shelled eggs three to four times annually (Schmidt, 2001). Eggs are oviposited in holes in the ground, require an incubation temperature of 25–30°C, and usually hatch in

120–180 days, depending on temperature (Schmidt, 2001). Females are known to store sperm (Schmidt, 2001), which insures some fertile eggs in future clutches long after copulation. The pastel green neonates are 55–75 mm TL and can reach 35–40 cm TL in one year (Schmidt, 2001).

We do not know the size and frequency of clutches for wild female *Chamaeleo calyptratus* in Florida, but because of abundant rainfall and food, we suspect that females may be more fecund here than in their native range. If clutch sizes are large and hatching rates high in Florida, this population might be difficult to eradicate. Tall grass in the vacant lot is occasionally mowed, undoubtedly killing some young *C. calyptratus*. Many adults and subadults are probably not found during searches because they are too high in trees or in dense vegetation. Additionally, small neonates are easily overlooked and could reproduce only four months later. Even if all neonates could be removed at any one time, multiple clutching by single females and long incubation times mean that different clutches of eggs could hatch sporadically and repopulate the area.

One neonate was found across the street behind a shopping center, indicating that a paved two-lane street did not present a barrier to either a gravid female or at least one neonate. Therefore, it seems likely that *Chamaeleo calyptratus* has already dispersed to adjacent neighborhoods north and west of the vacant lot. Major highways may preclude natural dispersal of *C. calyptratus* south and east of the vacant lot. Farther north, extensive wooded habitat is present along the Caloosahatchee River, but this estuarine habitat may be unsuitable for the species.

Chamaeleo calyptratus occurs in diverse habitats and environmental conditions in Saudi Arabia and Yemen (Fritz and Schütte, 1987; Meerman and Boomsma, 1987; Zari, 1993). *Chamaeleo calyptratus* prefers temperatures from 23° to 35°C (Schmidt, 2001), and this tolerance has enabled it to survive the hot summers and cool winters of southwestern Florida thus far. To escape low temperatures, individuals retreat into rock crevices or holes in the ground (Schmidt, 2001). In extremely hot conditions, *C. calyptratus* turns light colored and retreats into shade, sometimes cooling itself by gaping its mouth and panting (Schmidt, 2001). During drought conditions, individuals obtain moisture from dewdrops, prey, or feeding upon plants (Schmidt, 2001).

Chamaeleo calyptratus feeds primarily on insects, but its large size enables it to occasionally prey on small mammals and fledgling birds, making it a greater ecological threat to the native fauna than solely insectivorous exotic lizard species. *Chamaeleo calyptratus* is primarily a sit-and-wait predator that uses its independently moving eyes to spot prey, which is captured by rapidly protruding its sticky tongue with great accuracy to a distance of up to two times its SVL (Ott et al., 1998; Schmidt, 2001).

Additional populations of *Chamaeleo calyptratus* may become established in Florida in the future, particularly if reptile breeders or dealers release specimens in attempts to establish populations of this popular pet trade species for future exploitation. We recommend that monitoring of this population and its expansion continue, and if ecological impacts on native species are observed, efforts should be made to completely eradicate the population.

ACKNOWLEDGMENTS—We thank William B. Love, Chris S. Samuelson, Brooke L. Talley, Kristen L. Bell, and Tim Evans for field work; RobRoy MacInnes and William B. Love for information regarding *Chamaeleo calyptratus*; Kent Perkins and Barry Davis for plant identifications; Kenneth E. Barnett, Paul E. Moler, and D. Bruce Means for reviewing this manuscript.

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A RECORD OF A NONINDIGENOUS FISH, THE BLUE CATFISH (*ICTALURUS FURCATUS*: ICTALURIDAE), ILLEGALLY INTRODUCED INTO THE SUWANNEE RIVER, FLORIDA

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ABSTRACT: *I report on the first recorded specimen of the nonindigenous blue catfish (*Ictalurus furcatus*) from the Suwannee River in northern Florida. This represents an introduction far to the east of any known Florida populations. The specimen was captured on 23 January 2002 by hook-and-line in the vicinity of Rock Bluff along the border of Dixie and Gilchrist counties. Local anglers have reported additional catches, but these reports are unsubstantiated. Moreover, subsequent sampling for catfish by the Florida Fish and Wildlife Conservation Commission in 2002 and 2003 did not produce any additional specimens and therefore the persistence and reproduction of blue catfish in the Suwannee River is unconfirmed. The origin of this illegal introduction is unknown.*

Key Words: Blue catfish, *Ictalurus furcatus*, Suwannee River, Florida, non-indigenous, fish, introduced

NUMEROUS freshwater fishes have been illegally introduced into Florida. The majority are foreign species (i.e., exotic), are of tropical origin, and are largely confined to the southern portions of the state by cool winter temperatures in the rest of Florida (Shafland, 1996; Nico and Fuller, 1999). Hill (2002) provided a recent list of exotic fishes in Florida. However, northern Florida and the Florida Panhandle have relatively few nonindigenous fishes and these are mostly temperate transplants from other parts of the United States (Fuller et al., 1999).

Two predatory ictalurid catfishes of fisheries importance as well as ecological concern have been introduced into rivers of the Florida Panhandle. Both species have similar native distributions in the major river systems of the Mobile Basin, Mississippi River Basin, and the western Gulf of Mexico (Glodek, 1980a, b). The most well known is the flathead catfish (*Pylodictus olivaris*). This species preys upon sunfishes (Centrarchidae) and other catfish and its presence has been correlated with declines in redbreast sunfish (*Lepomis auritus*) and bullheads (*Ameiurus* spp.) in several river systems in the southeastern United States (Guier et al., 1984; Moser and Roberts, 1999). The other nonindigenous ictalurid, the blue catfish (*Ictalurus furcatus*), is established in the Escambia River in Florida and has been found in other Florida Panhandle rivers (FWC, 2003). Recent reports include the

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Apalachicola River (Cailteux, 2003) and the Alabama portion of the Chocta-whatchee River (i.e., upstream of Florida) (Metee et al., 1996).

On 23 January 2002, local anglers brought a catfish specimen to the Department of Fisheries and Aquatic Sciences, University of Florida, Gainesville, for identification. The catfish had been captured by hook-and-line from the Suwannee River near Rock Bluff along the border of Dixie and Gilchrist counties in northern Florida. The specimen was a relatively large, gray-blue catfish of 660 mm in standard length with 33 anal rays and a bilobed swim bladder and was identified as a blue catfish. This is the first record of a blue catfish from the Suwannee River system and represents an introduction far to the east of any known Florida populations.

Methods—Identification was based on meristic and morphological features detailed in Dunham and co-workers (1982), Etnier and Starnes (1993), Mettee and co-workers (1996), and Pflieger (1997). The specimen was deposited with the Florida Museum of Natural History (FLMNH) as catalog number UF 119654 (Robins, 2002). An examination of records at the FLMNH and the U.S. Geological Survey's (USGS) Nonindigenous Fishes Database (http://nas.er.usgs.gov/fishes/index.html) revealed no additional records from the Suwannee River system. The specimen was subsequently added into the USGS database (Fuller, 2003).

Results and Discussion—There have been a number of unsubstantiated reports of blue catfish in rivers of northern Florida (i.e., south and east of the Florida Panhandle systems that have known populations of blue catfish). I have examined putative blue catfish specimens on several occasions, mostly from the Oklawaha River system (St. Johns River drainage) and the Suwannee River drainage. However, these specimens were all white catfish (*Ameiurus catus*), a native species that superficially resembles the blue catfish. Moreover, many local anglers give the name "blue cat" or "blue catfish" to large specimens of white catfish. White catfish has a short anal fin with 21–26 (usually less than 24) anal rays whereas blue catfish has a long anal fin with 30 or more anal rays (Etnier and Starnes, 1993; Mettee et al., 1996).

The channel catfish (*Ictalurus punctatus*) is another native species that may be confused with blue catfish, especially if the specimen is large. However, the channel catfish lacks a bilobed swim bladder (Pflieger, 1997). Other distinguishing characteristics include the anal fin ray count (24-29 anal rays on channel catfish) and the shape of the anal fin (rounded margin in channel catfish versus straight margin in blue catfish) (Etnier and Starnes, 1993; Mettee et al., 1996).

Hybrids between channel catfish females and blue catfish males have been used in aquaculture in the southeastern United States (Masser and Dunham, 1998). This hybrid expresses paternal dominance in external appearance (i.e., body and anal fin shape) and swim bladder morphology and thus resembles the blue catfish (Dunham et al., 1982). Nevertheless, the channel catfish × blue catfish hybrid has a few scattered spots on the body; blue catfish lack spots (Dunham et al., 1982; Pflieger, 1997). Additionally, the swim bladder of the hybrid, although bilobed, has only a small posterior lobe (Dunham et al., 1982). Moreover, the only authorized use of blue catfish hybrids in Florida has been limited experimental work in the Florida Panhandle west of the Apalachicola River (Pouder, 2003).

The blue catfish is not established in the Suwannee River and there is no definitive evidence of reproduction. The anglers who collected the original specimen stated that previously they had caught catfishes of various sizes that closely resembled the specimen they brought to me. Subsequently, these anglers and a few others have reported additional blue catfish from the Rock Bluff area (Crumpton, 2003). However, given the common misidentification of native catfish as blue catfish by the public and the lack of any additional specimens produced by anglers, these reports must be considered as unsubstantiated. Moreover, personnel from the Florida Fish and Wildlife Conservation Commission intensively sampled the Suwannee River for catfish in July 2002 and 2003, including the area of Rock Bluff (Cailteux, 2003; Krummrich, 2003). Although thousands of catfish were collected (e.g., over 2300 in 2003), no additional blue catfish were discovered (Cailteux, 2003; Krummrich, 2003).

Like most illegal introductions, it is unlikely that the source of introduction will ever be known. Blue catfish is not native to Florida and its release is therefore subject to regulation. Since no permits have been issued authorizing the release of blue catfish into open Florida waters (Harrison, 2003), the introduction of this species represents an illegal act, punishable by law (FAC, 2003). This catfish, unlike channel catfish, is not stocked into recreational fishing ponds in Florida (Cichra, 2003). Additionally, channel catfish dominates the small Florida commercial catfish industry. An inquiry into the species of catfish listed on aquaculture certificates issued by the Florida Department of Agriculture and Consumer Services revealed no facilities certified for blue catfish production (Metcalf, 2003). Therefore, recreational ponds or aquaculture facilities are unlikely sources for the blue catfish specimen collected in the Suwannee River. Moreover, there are no freshwater connections between the Suwannee River and waters with blue catfish populations in Florida or Georgia.

In summary, a large specimen of the nonindigenous blue catfish was captured by an angler in the Suwannee River, Florida. This was the first confirmed blue catfish in Florida east of the Apalachicola River despite several putative specimens and unsubstantiated reports. The blue catfish should not be considered established in the Suwannee River and reproduction is unconfirmed. The introduction source is unknown.

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NITRATE AND PHOSPHATE UPTAKE BY DUCKWEED (*LEMNA MINOR* L.) USING TANDEM REACTORS

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ABSTRACT: *The use of the* Lemna minor *L. species of duckweed has proven to be effective in uptake and control of nitrogen and phosphorus levels. This research examines the uptake of nitrogen and phosphorus by* L. minor *under controlled environmental conditions using two 1600-mL Plexiglas* L. minor *growth reactors in tandem that permitted known concentrations of micronutrients to be added at known rates. Nutrient concentrations were measured until a depleted concentration state was reached (in a two-week period) A modified high nitrate Hillman's medium was used, and there was a 10% surface harvest every other day after the fourteenth day. This setup mimics the effluent wastewater going from one duckweed pond to another. The harvest improved the removal of nitrogen and phosphorus since the duckweed had room to grow.*

Key Words: mass balance, nutrient uptake, stormwater, sequential reactors

THERE is an increased interest in using aquatic plants, such as duckweed, in the treatment of contaminated surface waters and wastewaters. The use of this technology is of significant importance because it is a relatively cost- effective alternative to methods currently being used in the field of water treatment. Integrated algal and duckweed ponds have proven to be an effective means of controlling nutrient levels while not doing further damage to the environment (Van der Steen et al., 1998).

Five species of duckweed have proven to be effective in wastewater treatment (Bonomo et al., 1997), and one of these, *L. minor*, is the most common species in the state of Florida (Long and Lakela, 1976). The performance of common duckweed species on wastewater has been studied, and the results indicated that two species of duckweed, *L. minor*, *L. gibba*, and *Spirodela polyrhiza* proved to be the most effective in controlling nutrient levels (Vermaat and Hanif, 1998). Duckweed has proven to be important in the removal of nitrogen and phosphorus in domestic water systems (Körner and Vermaat, 1998).

Our previous research studied two approaches to the uptake of nutrients by *L. minor*: batch and continuous flow (Smith et al., 2004). With both methods, a continuous flow of medium entered the growth chamber from the feed reservoir. With the batch method, effluent from the reactor was recycled to the feed reservoir, and nutrient concentrations were measured until a depleted concentration state was

reached. This approach would be consistent with the passage of water through a re-circulating lemna pond system. The initial total phosphorus concentration was 320 ppm P (added as KH_2PO_4). Under these conditions, phosphorus was depleted in about fourteen days. The batch method was also applied to nitrate uptake, and it was found that the initial 5000 ppm NO_3-N was depleted in a fourteen-day period. In the continuous flow method, the reactors received an influent medium flow of 7 mL/min and operated at a liquid residence time of 229 minutes. The effluent from the growth reactor was discarded and fresh medium entered the reactor continuously. This approach would be consistent with the use of a duckweed pond without recycling to treat a nutrient- containing stream of water.

For the continuous flow method, a mass balance calculation on the reactors was performed using measurements of the mass of phosphorus and nitrogen added in the influent, the mass taken up in the growing duckweed biomass, and the mass exiting in the effluent. A mass balance for the continuous flow experiment with high nitrogen, low phosphorus medium (650 ppm N and 150 ppm P) indicated that 7% of the nitrogen and 10% of the phosphorus was removed by the plant uptake over the 14-day period of operation.

The present study examines the effect of tandem or sequential reactors in a continuous-flow mode. The system is thought to be analogous to a treatment train system in storm water runoff. Given the experience (Smith et al., 2004) with Hillman's growth medium, high-nitrogen Hillman's (enriched with KNO_3), and high phosphorus Hillman's (enriched with KH_2PO_4), we elected to examine the results with the second medium.

MATERIALS AND METHODS—*Growth chambers (reactors)*—These (Fig. 1), were made from of Plexiglas as described previously (Smith et al., 2004). The two reactors, arranged in tandem, accommodated the duckweed and the flow rate was controlled using two peristaltic flow pumps. The total volume of medium in the reactor was 1600 mL. We encased the sidewall and bottom of each reactor with black construction paper to limit the growth of algae, e.g. *Clamydomonas gloegama* Korschikoff, over the period of the study.

Culture conditions—All experiments and stock duckweed cultures were kept in a Phytotron, a controlled environment room (Environmental Growth Chambers, Chagrin Falls, OH) in the Department of Biology. Phytotron conditions were: constant temperature of 26 °C, 80% relative humidity, and a twelve-hour photoperiod with a light intensity of 190 $\mu E/m^2/sec$ measured by a LiCor model LI-185A photometer. The light intensity measured in the Phytotron room was equivalent to 33000 $kJ/m^2/day$ (16,500 $kJ/m^2/day$ for the 12-hour photoperiod), which was similar to the measured solar radiation of the months March and October (approximately 15,000 $kJ/m^2/day$) in the southeastern United States (Reifsnyder and Lull, 1965).

Duckweed (*Lemna minor* L.) was obtained from Carolina Biological Supply (Charlotte, NC). Stock duckweed was grown in plastic trays in a 100% Hillman growth medium (Hillman, 1959a,b). Growth medium was changed every three days to protect against loss of nutrients and the proliferation of algae. A modified (high nitrogen, low phosphorus, HNLP) Hillman growth medium, used in this project, was prepared by treating 15 L of Hillman growth medium with a 50% (w/w) spike of potassium nitrate, to gave a total nitrogen level of 721 ppm N and a total phosphorus concentration of 155 ppm P.

Tandem reactor experiments—Medium in a 15 L Pyrex carboy and the reactors were autoclaved at 60 psi and a temperature of 115 °C for 90 minutes. When the medium was brought to the Phytotron room, a black plastic bag covered the medium during the study to prevent the growth of algae. Autoclaved

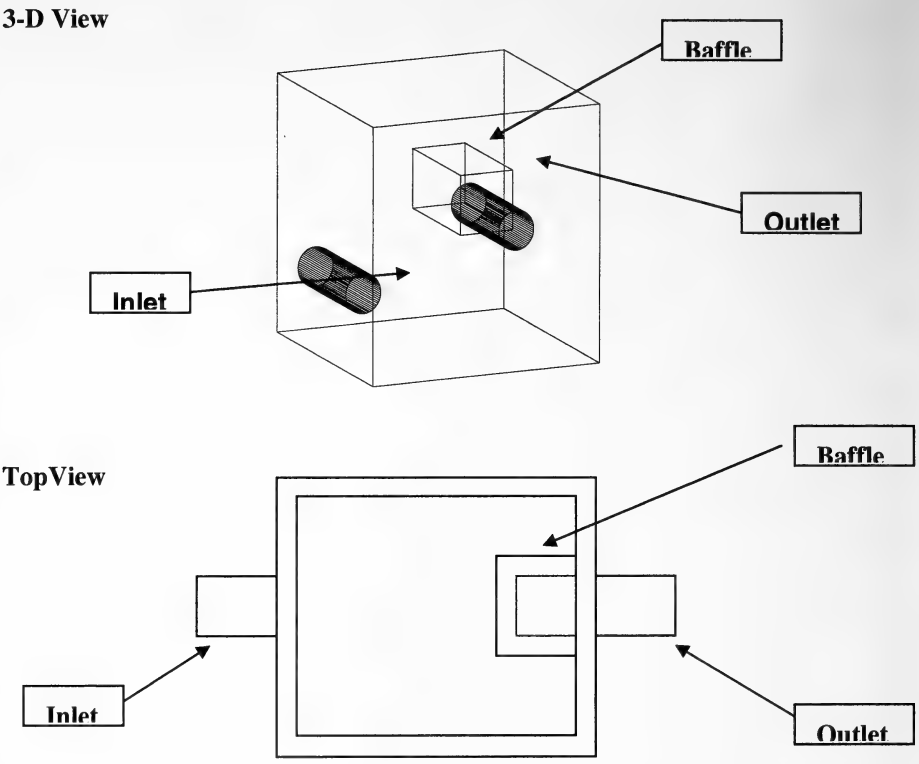

FIG. 1. Schematic representation of *L. minor* growth reactors. Reactor dimensions were 15.2 × 8.3 × 12.7 cm.

medium was pumped into the reactor using a peristaltic pump (Cole-Parmer Model 07554–80) with a pump head (07518–12) using Tygon (LFL L/S® 25) tubing. Then the duckweed was transferred from the plastic trays and placed in Plexiglas growth reactors (Fig. 1). In all the studies, the medium was monitored for changes in concentration of nutrients. The biomass in each growth reactor was determined at the start of the study and at the completion of the experiment using a previously described procedure (Smith et al., 2004). Each time the reactor was initially filled with duckweed, the reactor surface was mixed to spread the duckweed fronds uniformly over the reactor surface area, and a separate scoop of stock duckweed was collected and analyzed to determine the starting duckweed biomass per reactor surface area. This separate scoop was weighed fresh and dry and then multiplied by the number of scoops it took to fill the surface of the reactor of that particular study.

Two lemna reactors were arranged in series such that the effluent of the first reactor became the influent of the second. We used a flow rate of 7 mL/ min, so that water replacement time was 229 min, and fresh medium was made daily. One 40- mL water sample was taken every other day from three sampling points (total of three 40-mL water samples) using a three-way stopcock placed in the influent hose of the first reactor, a second three-way stopcock placed in the effluent hose of the first reactor, and the third sample came from the effluent of Reactor Two. A peristaltic pump placed prior to the first reactor pumped medium into the reactor. A second peristaltic pump between the second three-way stopcock and the second reactor prevented overflow in the first reactor overflow (flow rate of 10 mL/ min) and forced medium through the second half of this system. A 10% surface area harvest was started on the fifteenth day and was continued every other day until the end of the study, day 55. Phosphorus and nitrogen analyses were performed on the water samples.

Analyses—A Hach total phosphorus kit (model PO-24, Hach Company, P.O. Box 389, Loveland, CO EPA method 353.3) and Hach kit (Model Pl-14; EPA method 365.2) for nitrate analysis were used. The kit instructions were followed with only slight modification (Smith et al., 2004). Water samples (40 mL) were analyzed in triplicate and the mean and standard deviation were recorded. The total aqueous phosphate and nitrate values were converted to total phosphorus and nitrogen.

Fresh weights for duckweed samples was determined as before (Smith et al., 2004). After the fresh weight was determined, the duckweed sample was placed in a test tube and transferred to an oven(56° C) for 24 hr. Then the sample was cooled to room temperature and weighed. The relationship between dry weight (D.W.) and fresh weight (F. W.) was evaluated using 15 samples (Eqn. 1)

$$\text{D.W.} = 0.0566 * (\text{F.W.}) + 0.0015 \quad (1)$$

Here, D.W. = Dry weight (in g.) and F.W. = Fresh weight (in g.)

Fresh weight was also related to the frond count, using appropriate data (Smith et al., 2004) as indicated (Eqn. 2)

$$\text{Fresh weight} = a + b * (\text{fronds}) \quad (2)$$

The fresh weight of scooped duckweed was then determined as described above. After weighing, the duckweed was returned to the *L. minor* growth reactor. It took five scoops to completely cover the surface of the each of the reactors, where the duckweed formed a green mat. It was necessary to calculate the fresh weight for seeding the reactors and then converting it into dry weight (Eqn. 1, 2).

When analyzing for nitrogen or phosphorus in biomass, the dry weight of the plant matter was first recorded. The dried plant sample was then digested with dilute (7.5 *M*) H_2SO_4, allowed to stand for a day, then neutralized with 7.5 *M* aqueous NaOH. The mixture was then filtered using Whatman GF/A filter paper to remove any undigested plant particles. The filtrate volume was recorded, and the sample was analyzed for the nitrate and phosphate.

Results and Discussion—*Analyses*—Nutrient (orthophosphate and nitrate) analyses were routinely performed (Table 1). Mean and relative standard deviations were calculated as a means of evaluating precision. For example, for phosphate the relative standard deviation of the mean was 2.5%, while the corresponding value for nitrate was 2.3%, In addition, the percent recovery measured for phosphate and nitrate analyses (Smith et al., 2004) was 97.8–103% (P) and 90–99%, (N). The results from all the experiments with single reactors in a previous study (Smith et al., 2004) showed that nitrate and phosphate levels decreased over the period of the investigation (14 days).

Tandem-reactor experiments—These experiments can be compared with previous ones, involving a single reactor. In those experiments (Smith et al., 2004), the nutrient concentration began to level off, or reach a steady state, by the fourteenth day. In the tandem study, however, there was a 10% harvest beginning the fourteenth day, so that the duckweed did not reach this steady state condition. The plants grew more, and thus had the ability to remove more nutrients. This study started with around 85% duckweed cover of each reactor, and by the fourteenth day, there was a 100% surface cover of duckweed in both reactors. During the first few harvests, the duckweed cover was thicker than the later harvests where the duckweed grew at a more consistent rate. Furthermore, a few days after the initial harvests, the duckweed adjusted to open growing space, which was reflected in a spike or variance in the general downward curves around day 20.

TABLE 1. Harvest data of each reactor and nitrogen and phosphorus content in the harvested dry weight (dw) of the tandem study.

Day	Reactor	DW, g	N, g	%N in biomass	P, g	%P in biomass
15	1	0.050	0.00081	1.6	0.00008	0.17
	2	0.062	0.00055	0.89	0.00014	0.2
17	1	0.050	0.0045	9.0	0.00004	0.07
	2	0.060	0.0035	5.8	0.00004	0.06
19	1	0.062	0.00097	1.6	0.0001	0.1
	2	0.10	0.0027	2.7	0.00007	0.07
21	1	0.0545	0.00572	10.5	0.000104	0.19
	2	0.036	0.00128	3.6	0.0000653	0.18
23	1	0.0944	0.00342	3.6	0.0000930	0.10
	2	0.0573	0.0006	1.0	0.0000352	0.06
25	1	0.121	0.000915	0.8	0.0000261	0.02
	2	0.0512	0.000704	1.4	0.0000261	0.05
27	1	0.0632	0.000893	1.4	0.0000473	0.07
	2	0.0396	0.000813	2.1	0.0000457	0.12
29	1	0.0678	0.001373	2.0	0.0000509	0.08
	2	0.0818	0.001487	1.8	0.0000416	0.05
31	1	0.0612	0.00143	2.34	0.0000478	0.08
	2	0.0412	0.000985	2.39	0.0000488	0.12
33	1	0.0652	0.000893	1.37	0.0000510	0.08
	2	0.0423	0.000856	2.02	0.0000430	0.10
35	1	0.05	0.001375	2.75	0.0000403	0.08
	2	0.041	0.001542	3.76	0.0000380	0.09
37	1	0.0549	0.000759	1.38	0.0000358	0.07
	2	0.0511	0.0012	2.35	0.0000389	0.08
39	1	0.0598	0.000746	1.25	0.0000473	0.08
	2	0.0549	0.001589	2.89	0.0000410	0.07
41	1	0.0678	0.000856	1.26	0.0000475	0.07
	2	0.0818	0.000678	0.83	0.0000414	0.05
43	1	0.05	0.001375	2.75	0.0000454	0.09
	2	0.041	0.00103	2.51	0.0000427	0.10
45	1	0.0428	0.000759	1.77	0.0000471	0.11
	2	0.0511	0.0012	2.35	0.0000348	0.07
47	1	0.0896	0.000856	0.96	0.0000470	0.05
	2	0.0468	0.000973	2.08	0.0000490	0.10
49	1	0.0543	0.001375	2.53	0.0000520	0.10
	2	0.058	0.00123	2.12	0.0000434	0.07
51	1	0.064	0.000856	1.34	0.0000514	0.08
	2	0.0989	0.001469	1.49	0.0000442	0.04
53	1	0.079	0.001326	1.68	0.0000471	0.06
	2	0.062	0.00123	1.98	0.0000479	0.08
55	1	0.0746	0.001375	1.84	0.0000528	0.07
	2	0.033	0.0006	1.82	0.0000446	0.14
Literature*				0.8–7.8		0.03–2.8

* Landolt and R. Kandeler (1987).

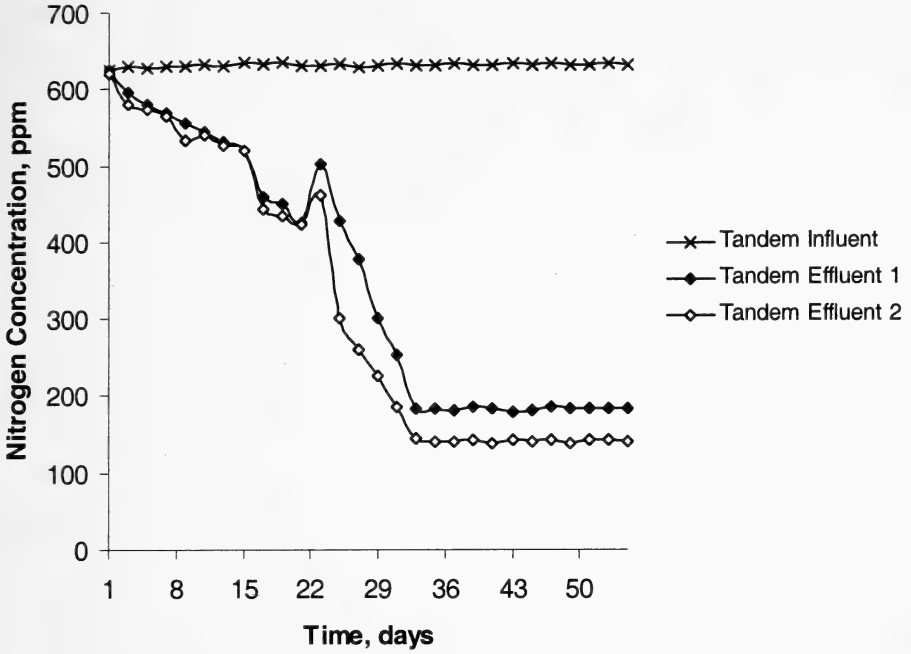

FIG. 2. Nitrogen concentration of medium vs. time for tandem experiment. Here x corresponds to the medium concentration coming to Reactor One. The nitrogen concentration (as ppm) of the solution leaving Reactor one (closed diamonds) and Reactor Two (open diamonds) is also given.

Table 1 and associated plots (Fig. 2) indicate a general decrease in phosphorus and nitrogen in both reactors with time with some variations. After the fourteenth day, the duckweed did not remove the expected amount of phosphorus from the medium. Interestingly, the nitrogen concentration of the medium sharply decreased after the first harvest (Fig. 2). Nitrogen was not removed well during days 15–23; this is ascribed to readjustment of the open space in the reactor. After this readjustment period, lasting about three days, the duckweed in the first reactor again removed ample nutrients from the medium as may be seen from where the plotted curve continued the decreasing slope (Fig. 2). On the other hand, duckweed in the second reactor, having less available nitrogen, did not grow as aggressively. With a month into the experiment, and a full two weeks of harvesting every other day, the duckweed reached a steady state.

Nutrient-removal analysis—Finding the uptake of nutrients in the two tandem reactors is the significant aspect of this project. A key step is converting the concentration of the nutrients in the medium into a more understandable form of how many grams the duckweed removed. We used a variation on mass balance equations (Eqn. 3) taking appropriate data (Table 1) for nitrogen concentrations (for example)

$$\text{Uptake} = (\text{Influent Concentration} - \text{Effluent Concentration}) * \text{Volume of water} \quad (3)$$

in order to calculate the amount of removed (Table 1).

The following information was obtained. Reactor One absorbed a steady amount of nitrogen throughout the entire study (55 days), but the nitrogen absorption in the second reactor was less because of the lower concentration of nitrogen in the influent of Reactor Two and only having 229 minutes to remove it, decreased the opportunity for duckweed to remove nitrogen. Nevertheless, Reactor Two still removed nitrogen and maintained around a 100% duckweed water surface area cover during the study. Reactor Two slightly lagged behind Reactor One with the initial harvesting. Eventually both reactors attained a steady state of nitrogen absorption by day 30. Even though Reactor Two had much lower nitrogen absorption, it was greater after the harvest than before, i.e., 0.18 vs. 0.05 g nitrogen per day. Clearly, harvesting duckweed and providing the remaining with more available free space would be more beneficial in removing nitrogen than leaving it alone after a steady state is reached, and would point to the need for technical assistance in arranging for harvesting in a stream or multi-pond situation.

Average nitrogen concentrations were calculated during the steady state period (days 35 to 55). There was a noticeable increase of nitrogen removal in Reactor One compared with Reactor Two, 71% (of 631 ppm N) vs. 23% (of 183 ppm). In the same period, the average removal of phosphorus in Reactor One was 26% (of 151 ppm), in Reactor Two was 38% (of 111 ppm). During this time, both of the two duckweed cultures were acclimated to the nitrogen and phosphorus in their influent.

In the harvested duckweed, the nitrogen content was around 1–3% of dry weight biomass (Table 1). Although a few values were outside of this range, for example, days 17 and 21 Reactor One had a 9 and 10.5 percent of nitrogen in the biomass (dry weight). However on these days the duckweed was in the process or of attaining a steady- state condition. All other values were consistent with the range reported by Landolt and Kandeler (1987), i.e., a duckweed nitrogen content between 0.8–7.8%. The duckweed apparently had ample phosphorus to grow to replace the harvested duckweed; the percent of phosphorus of dry weight biomass of the harvested samples did not exceed 0.19% (Table 1), well within Landolt and Kandeler's (1987) range of 0.03–2.8% phosphorus per biomass.

This study indicated the potential success of a tandem arrangement for managing storm water runoff through treatment with duckweed in confined areas. The study also indicates the need for appropriate monitoring and harvesting. This study provides data for the rate of uptake, and the indication that rapid flow would not lead to an effective removal of nitrogen or phosphorus, but in a stream situation, e.g. a creek, it might be necessary to have a longer reach of duckweed areas.

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EFFECT OF CHEMICAL MATRIX ON HUMIC ACID AGGREGATES

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ABSTRACT: *Using laser diffraction, we measured the aggregation of humic acid (HA) and the impact various chemical conditions (pH, ionic strength, lanthanides, transition metals, anions, etc.) have on the average size of HA and found that large aggregates persist over a range of conditions. Second, we examined the kinetics of aggregation and precipitation over forty days and found a critical size is reached before precipitation occurs.*

Key Words: humic acid, humic substances, aggregation, laser diffraction, Suwannee River

RESEARCH with humic substances (HS) has been an ongoing endeavor for a variety of science and engineering disciplines over the past century. Their perceived role in environmental health and technology issues has been growing as the ability of HS to solubilize, bind, and transport various organics (herbicides, pesticides, etc.) and inorganics (actinides, heavy metals, etc.) is better understood. Subsequently, HS have been divided into three groups: fulvic acid (FA), which is soluble at all pHs, humic acid (HA), which precipitates out of aqueous solution below a pH of 2.0, and humin, the insoluble, nonpolar organic component. HS are generally attributed to the molecular constituents of plant and animal decay in nature, but may undergo further structural changes due to exposure to UV light from the sun, microbial decay, various oxidizing agents such as oxygen, and other environmental changes such as metal binding, pH shifts, and changes in ionic strength. HS play an important role in environmental chemistry. For example, they can bind and transport metals through the environment, solubilize nonpolar compounds (i.e. herbicides, pesticides, and petroleum products), fertilize soil, buffer soil and water, impact dissolved oxygen levels in the aqueous phase, etc. (Suffet and MacCarthy,1989; Davies and Ghabbour, 2000; Davies et al., 1999; Klavins et al., 1999). The international standard often used for HA is taken from the Suwannee River in Fargo, Georgia (Dixon et al., 1999; Leenheer et al., 1995; Averett, 1994).

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Past work in this laboratory with HS has included using multiangle laser light scattering (MALLS) to measure the average size and molar mass of HA aggregations under one set of conditions (Manning et al., 2000) to a variety of thermodynamic type studies (Hayes et al., 1995; Fiskus and Manning, 1998; Gravely and Manning, 1995). It has been proposed by Guetzloff and Rice (1994) and supported by experimental data that HS can form micelles, which subsequently solubilize DDT. Specifically, humics have a large, nonpolar component that aggregates in solution, providing a region that is chemically and physically compatible for larger nonpolar organic species. Our research has shown that HA aggregates, from a specific source, have an average size of 0.4 μm and a molar mass of 10^9 D. What should be emphasized is that HA is an aggregation of many molecules present over a wide range of concentrations, including aliphatic and aromatic structures, multiply substituted carboxylates (Xing and Chen, 1999; Frimmel and Christman, 1988), amino acids and peptides (Tarr et al., 2001; Sommerville and Preston; 2001), sugars (Clapp and Hayes, 1999), cellulose and lignin fractions (Lehtonen et al., 2000), and functional groups including thiols, amines, and phenols (Lin et al., 2001). Techniques routinely used to characterize HS include CHNOS analysis (Meyers and Lallier-Verges, 1999; Calace et al., 1995; Shindo, 1991), ^{13}C NMR (Klucakova et al., 2000; Smernik and Oades, 2001), pyrolysis GC-MS (Davies et al., 2001; Lu et al., 2001; Gonzalez-Vila et al., 2001), UV/VIS (Langhals et al., 2000; Maia et al., 2001), fluorescence (Esteves and Duarte, 2001; Filippova et al., 2001), X-Ray analysis (Monteil-Rivera et al., 2000; Bubert et al., 2000), size exclusion chromatography (Piccolo et al., 2001; Aguer et al., 2001), and FT-IR (Mueller et al., 2000; Francioso et al., 1998; Spaccini et al., 1998). A large number of binding studies involving different forms of HS from different global locations have also been conducted. These studies include various elemental binding (i.e. Ca^{2+}, Cu^{2+}, lanthanides, actinides, etc.) and the trapping of various organic compounds such as herbicides and pesticides (Janik et al., 1998; Pompe et al., 2000; Kogut and Voelker, 2001; Gu et al., 2001; Gondar et al., 2000; Christl and Kretzschmar, 2001(b); Peuravouri, 2001). Past research in this laboratory examined binding of fluoride and calcium to HA (Hayes et al., 1995; Fiskus and Manning, 1998; Gravely and Manning, 1995) and showed that cations and anions can bind via site (i.e. ionic bond) or territorial (trapped in large structure) mechanisms. In this study, our aim is to better understand the aggregation process of humics and, subsequently, better understand their dynamics in binding and transporting various species in the environment.

Materials and Methods—Aldrich HA [cat. #H1,675-21] was used for all laser diffraction studies and was dissolved directly into DIUF water. Typically solutions were sonicated for 30 seconds to accelerate the dissolving process. Humic acids concentrations in systems were its concentration was held constant (i.e. ionic strength, pH, binding to Cu(II), Ln(III)'s, etc.) was set at 10 ppm. Metal concentrations in binding studies (i.e. Cu(II), Ln(II)'s) were on the order of 10^{-7} to 10^{-5} M. All lanthanides used were purchased from Aldrich as hydrated nitrates; cerium (III) nitrate hexahydrate (Aldrich 20,299-1), praseodynium (III) nitrate hexahydrate (Aldrich, 20,513-3), neodynium (III) nitrate hexahydrate (Aldrich, 28,917-5), samaruim (III) nitrate hexahydrate (Aldrich, 29,812-3), europium (III) nitrate pentahydrate (Aldrich, 20,791-8), gadolinium (III) nitrate pentahydrate (Aldrich 21,719-0), terbium (III) nitrate

pentahydrate (Aldrich, 32,594-5), dysdrosium (III) nitrate pentahydrate (Aldrich, 29,815-8), holium (III) nitrate pentahydrate (Aldrich, 32,573-2), erbium (III) nitrate pentahydrate (Aldrich, 29,816-6), thulium (III) nitrate pentahydrate (Aldrich, 29,816-6), lutetium (II) nitrate hydrate (Aldrich, 43,642-9). The pH of solutions were adjusted with 0.1 N HCl or 0.1 N NaOH. With the exception of studies that involved measuring aggregate size over a range of $[H^+]$ values, solution pH's were held in the pH 4-5 range in order to avoid hydrolysis of cations. The use of an additional buffer was avoided in order to minimize any interactions that might impact aggregation. We allowed the humic acid, which has carboxylates with pK_a's in the 4.5 range, to buffer themselves. Laser diffraction measurements were made with a Shimadzu 3001 laser diffraction instrument and a Wyatt Technology MALLS (MultiAngle Laser Light Scattering) instrument. With both laser systems, software provided by the vendor was used to perform the calculations. The Wyatt MALLS system was used on solutions where particles sizes were under 100 nm (i.e. fig. 2a,b) and the Shimadzu system was used when particle sizes were in the 0.1 to 2000 micrometer range. For the Shimadzu instrument, approximately 280 milliliter of solution were used in each experiment. For the Wyatt MALLS instrument, approximately 15 mls of solution was used in each measurement. For the long-term kinetics studies (>30 days), ten (10) liter carboys were set up with the respective solutions. The amount used for particle size testing was removed by a 100-milliliter pipet with all attempts made not to disturb the system. A total of fifteen carboys were set up to test the impact that various anions, cations, pH's and salt concentrations had on the average particle size of humic acid. Diionized Ultrafiltered (DIUF, 18 Mohm) water was used to make all solutions.

RESULTS—The first four sections outline the results and discussion of experiments studying the aggregation of HA as a function of its aqueous phase chemical matrix. The last section outlines the results of long-term (40 days) kinetic studies, measuring the aggregation and precipitation of humics in different chemical matrixes.

Copper(II) binding studies—Typical of several divalent transition metals, $Cu^{2+}(aq)$, as shown by Kogut and Voelker (2001) and Schmitt and co-workers (2001), can bind HA by electrostatic and covalent bonds. In this work, we made two sets of measurements to better understand the role that divalent copper plays in the aggregation and precipitation of HA in a slightly acidic environment. This study measured the effects that the Cu^{2+} concentration had on the particle size distribution. We measured the total number of particles as a function of size. This approach involved measuring the distribution of mass as a function of size. In a typical system, it might be possible to have a significant number of smaller particles but have a majority of mass reside in a few very large particles.

Figure 1a illustrates that most of the particles are quite small (0.35 μm) over a fairly wide $[Cu^{2+}]$ range. Figure 1b demonstrates that the majority of the volume or mass of the HA is contained in a few larger particles that are in the 10–30 μm range. As shown (Fig.1b), the mean size of the Cu^{2+}-HA particles remained relatively constant in the 7–10 μm range, but the median size decreased as the Cu^{2+} concentration increased until the HA size stabilized at approximately 10 μm. The modal size remained constant until a Cu^{2+} concentration of 50×10^{-7} M was reached; then the modal size increased slightly from approximately 24 μm to 28 μm. The mean, median, and mode had standard deviations in the 0.5 to 0.6 μm range. In this experiment, the pH was maintained in the 5.7 to 5.9 range. While Cu^{2+} will undoubtedly bind functional groups within HA, it does not play

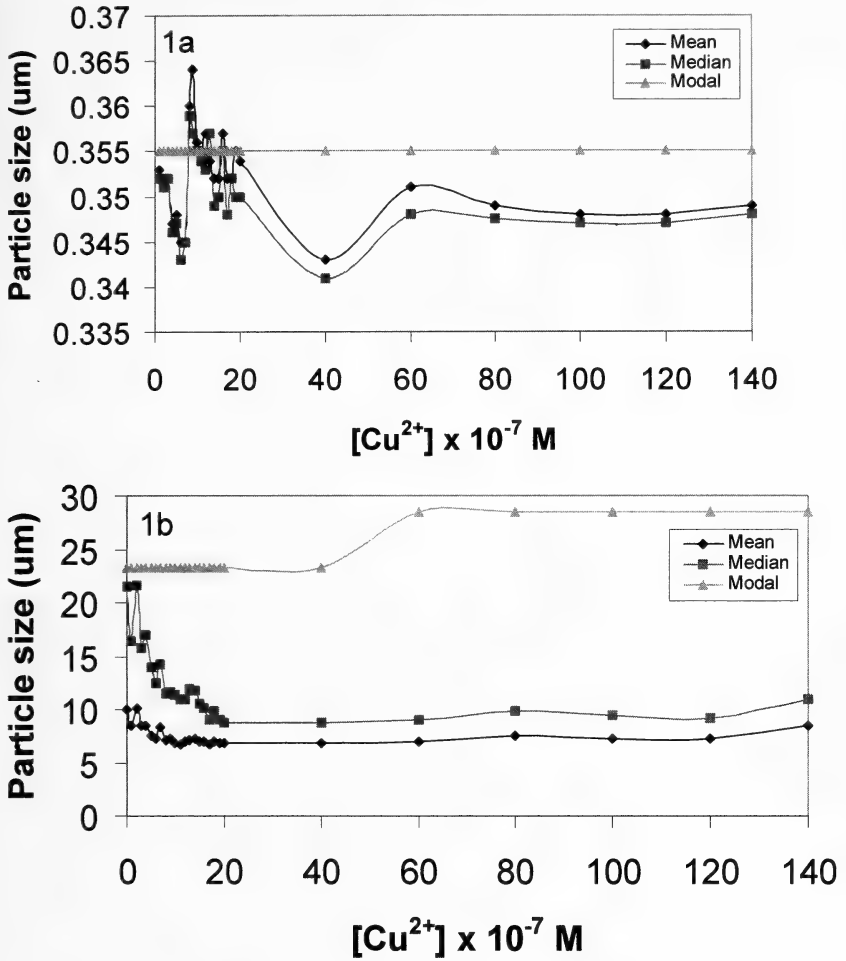


FIG. 1a,b. The impact that increasing the $[Cu^{2+}]$ has on the mean, median, and mode of the particle size, measured by number (a) and volume (b), is minimal.

a significant role in determining the size of the aggregate or the distribution between the relatively large particles (25 μm) and the smaller ones (0.30 μm). Because HA is made up of a wide range of smaller molecules (including amino acids and simple carboxylic acids), it is proposed that with this particular HA sample, while the Cu^{2+} will bind one of two sites (amines, carboxylates), it does not cause the HA structure to contract.

Figures 2a,b show data for a specific point on the plots shown in Figures 1a,b. Figure 2a shows the particle distribution by volume, or the number of particles or aggregates at each particular diameter. Approximately 80% of the HA mass or volume is contained in aggregates between 3 and 50 μm in size. Figure 2b shows the diffraction measurement of the same Cu^{2+}-HA system when the normalized particle

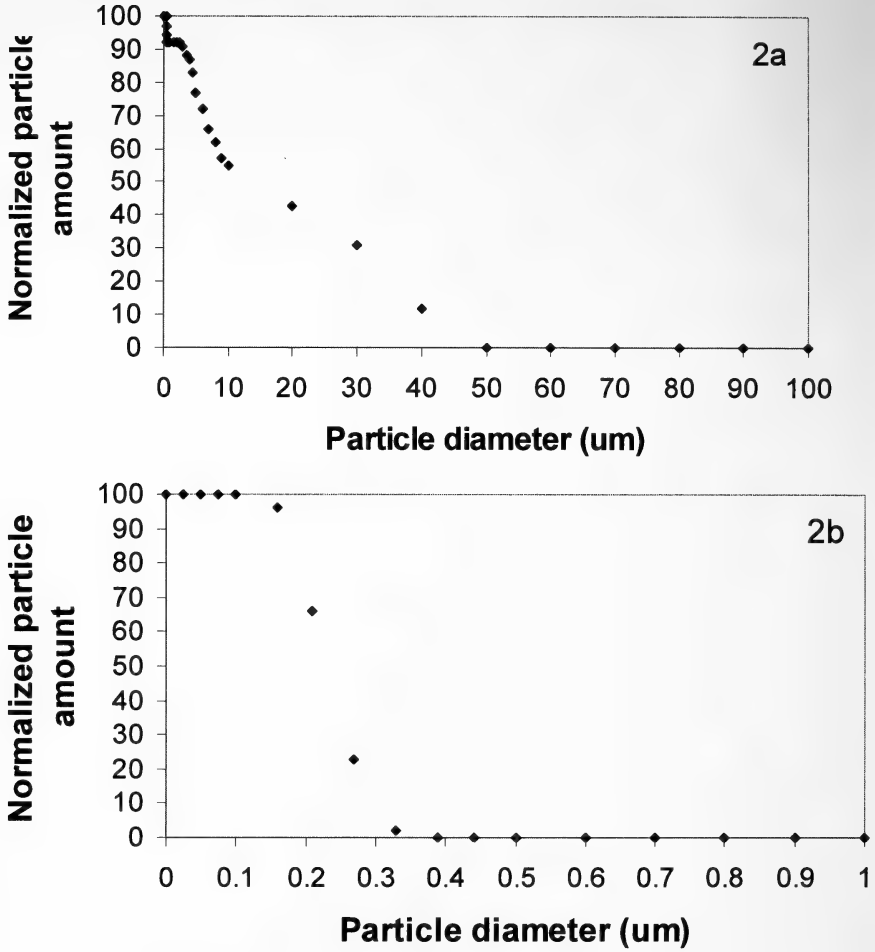


FIG. 2a,b. The particle size distribution as a function of volume (a) and number (b).

amount is measured as a function of the number of particles present. While less than 10% of the total mass or volume of the humic aggregates is contained in particles less than 0.5 μm in diameter, more than 99.9% of the number of particles have this diameter. This illustrates that while there are many smaller particles, the majority of HS are found in a few larger aggregates.

Ionic strength—The effect of increasing the ionic strength (using NaCl) from 0 to 1.0 on HA aggregation was investigated. The impact that ionic strength has on HA and FA in terms of their physical and chemical parameters has been measured (Christl and Kretzschmar, 2001(a,b); Peuravouri, 2001; Antonelli et al., 2001; Schmitt et al., 2001; Tombacz et al., 2000; Carballeira et al., 1999). We did not consider any of the background ions associated with HA salt; only the salt added

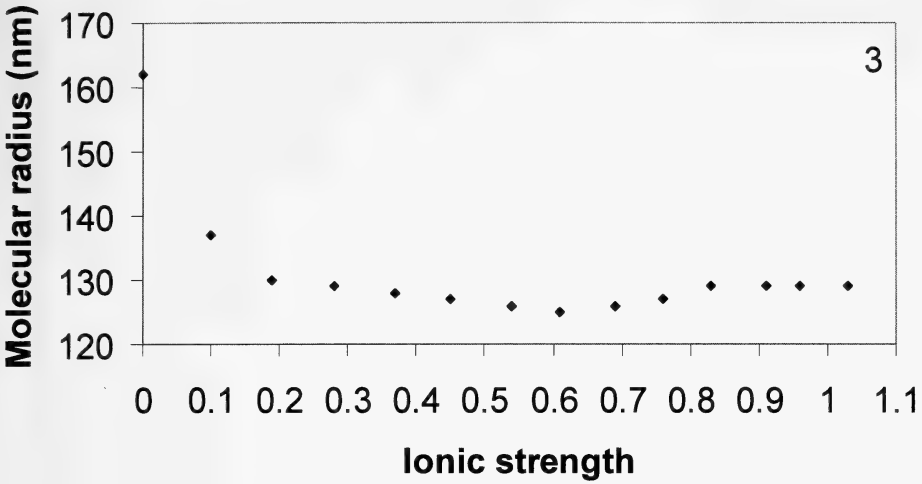


FIG. 3. The average radius (nm) of the HA aggregate as a function of ionic strength.

during the experiment. The results illustrated (Fig. 3) show that, at a pH of 7.0, as the ionic strength was increased, the root mean squared (rms) radius was decreased from 160 nm to 130 nm. These values had a typical standard deviation of 5–6 nm. Most of the change in radius occurred in the low ionic strength range. We propose that, through a series of weak electrostatic interactions dominated by outer sphere complexation involving the Na^+ and Cl^- ions and charged species (i.e. R-COO^-, R-NH_3^+) or significant dipoles (phenol, alcohol, etc.) present in the HA, a constriction of the HA structure takes place. These measurements are important for a foundation of our model because, as we travel through the Suwannee River basin, we encounter a range of water conditions: low ionic strength water flowing from springs, high ionic strength water from the Gulf of Mexico, and extremely hard water that forms in isolated limestone lined pools. These data (Fig. 3) show that aggregates will form over a wide range of salinities.

Variations in pH and HA concentration—Various acidity characteristics of HAs (pK_a determinations, effect of pH on metal binding) have been reported in the literature (Engebretson and Von Wandruszka, 1997; Senesi et al., 1997; Falzoni et al., 1998; Dai et al., 1996; Gulmini et al., 1996; Leenheer et al., 1995). Figure 4 presents the results of experiments that measured the effect of varying $[H^+]$ has on the molecular radius of HA. In the same way that increasing ion concentration with NaCl caused the structure to contract, we observed the same effect with increasing $[H^+]$. In the basic pH range (>7), many of the functional groups are either completely deprotonated (carboxylates, amines) or partially deprotonated (phenol); the structures appear to unravel (larger radius). This can be attributed to a minimal number of hydrogen bonds between functional groups. As the pH decreases, the protonation of the various functional groups increases, causing a substantial

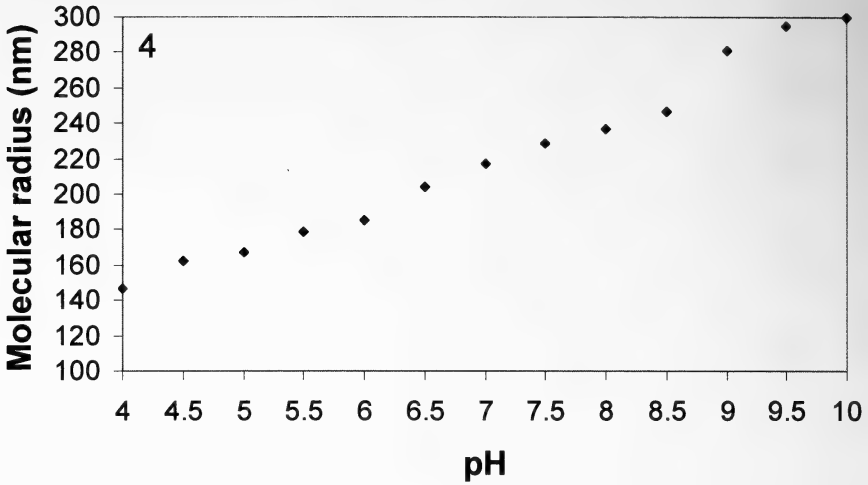
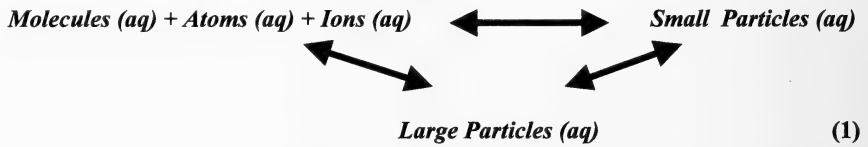


FIG. 4. The effect that pH has on the average radius of the HA aggregate.

increase in the number of hydrogen bonds, therefore resulting in a structural contraction.

The impact of HA concentration (g/L) on its own size has also been studied and reported in the literature (Osterberg et al., 1993; Barak and Chen, 1992; De Wit et al., 1993). For this work, we held the pH at approximately 6.0, and the concentration of HA in deionized ultra filtered (DIUF) water was increased from 0.3 to 40 ppm by adding the solid HA salt to the aqueous phase and adjusting pH with dilute NaOH. As the HA concentration increased, the average rms radius of the aggregate increased (Fig. 5). This points to a complex equilibrium (Eqn. 1)



(1)

The aggregate grew larger with concentration until precipitation occurred (Fig. 5). There appeared to be a critical mass or saturation point of the 0.30 and 25 μm particles that was reached before rapid precipitation occurred, indicating that 0.30 μm particles aggregate to form 25 μm particles that subsequently precipitate. This aggregation and precipitation trend is similar to observations in the Cu^{2+} experiments outlined earlier. While the exact size of particles may change with the source of HA utilized or the chemical and physical conditions (pH, ionic strength, temperature, etc.) selected, we observed the same trend in terms of small water-soluble aggregates combining to form large aggregates that precipitate. Particles or aggregates that are hundreds of nanometers in size appeared stable in solution (over minutes) until some change (pH, ionic strength, etc.) induced precipitation. These smaller particles combined to form larger aggregates in the

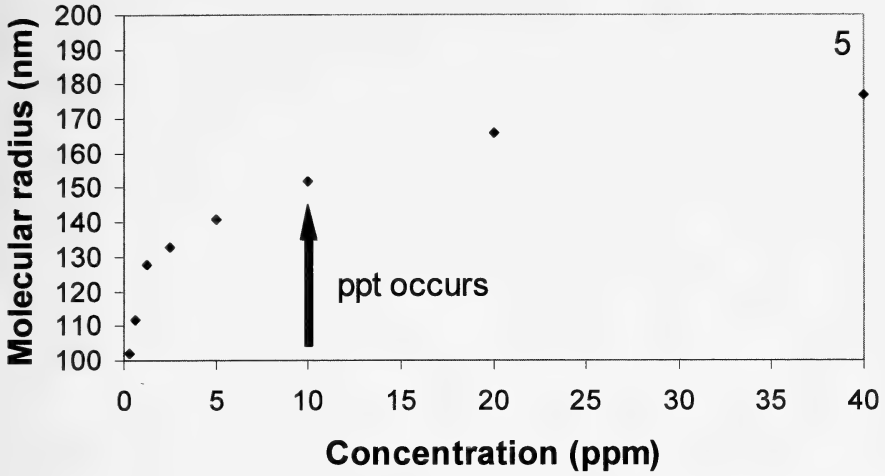


FIG. 5. The molecular radius of HA as a function of HS concentration. Usually, we observed a precipitate in a few minutes when HA was in the 10–20 ppm range.

3–100 μm range that fall out of solution. We believe it is these aggregates that help bind, concentrate, and transport natural products (NP) in the environment.

Lanthanides—While the lanthanides are the most obscure group on the periodic table in terms of research endeavors and commercial applications, there have been some studies by Ozaki and co-workers (2000), Dierckx and co-workers (1994), and Moulin and co-workers (1992), measuring the binding of the trivalent metals in the aqueous phase to various HS components. Lanthanides, which are trivalent and bind primarily by ionic bonding in solution, increase in charge density (Z/r) from lanthanum (Z = 57) to lutetium (Z = 71). We measured the average aggregate radius for twelve of the lanthanides (excluding Pm, Tm, and Lu). These are shown in Figure 6.

We believed a correlation between the average Ln(III) ionic radius and the size of the humic aggregate might exist, but instead found the aggregate sizes in the 40–80 nm range did not follow these trends, as illustrated (Fig. 6). Lanthanide ions, which have coordination numbers between seven and nine, clearly impact HA aggregates to a greater extent than divalent copper ions do. We propose the following explanation for this observation. When the Cu^{2+} ion binds to HA, it binds primarily to amine groups, whereas the lanthanide ions bind by electrostatic attraction to carboxylate groups (Ln(III)-COO^-). The stability constant for Cu^{2+}–NH_3 ($\log\beta = 4.12$) is larger than Cu^{2+}-acetate ($\log\beta = 1.82$). The lanthanides have a negligible interaction with ammonia, but do interact with acetates (average $\log\beta = 2.0$–2.4 for Ln(III)-CH_3COO^-). Because nitrogen is in fairly low abundance in humics (see Table 1), Cu^{2+} binding of humics will be site specific and not impact the entire HA structure. Humics, which are rich in carboxylates, can have several

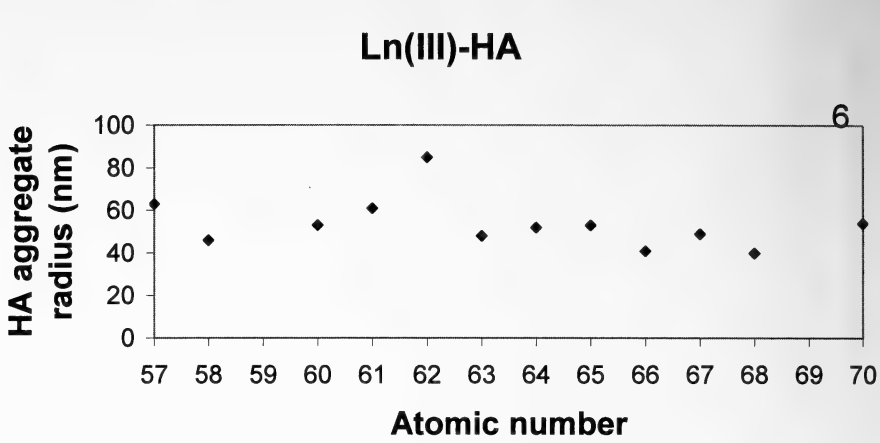


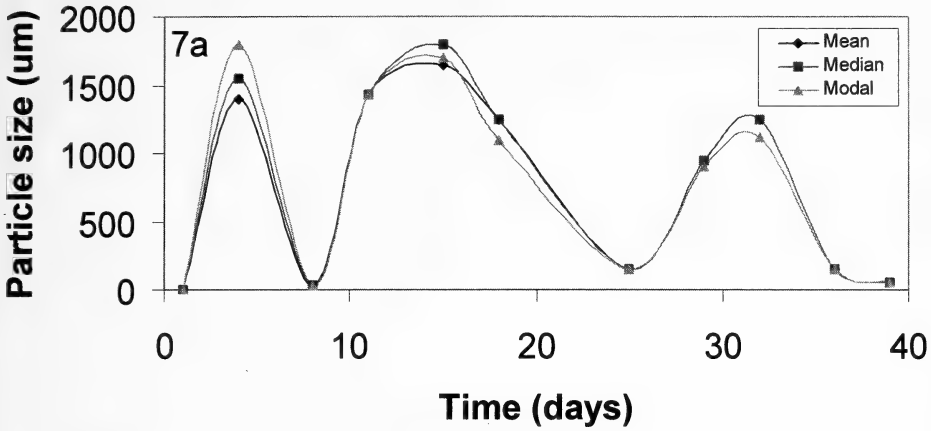
FIG. 6. The result of the average radius of the HA aggregate in a 10 ppm solution in the presence of 10^{-6} M Ln(III) at a pH of 5.0. The numbers correspond to lanthanides from La^{3+} (Z = 57) to Yb^{3+} (Z = 70), excluding Pm (Z = 59), Tm (Z = 69), and Lu (Z = 71).

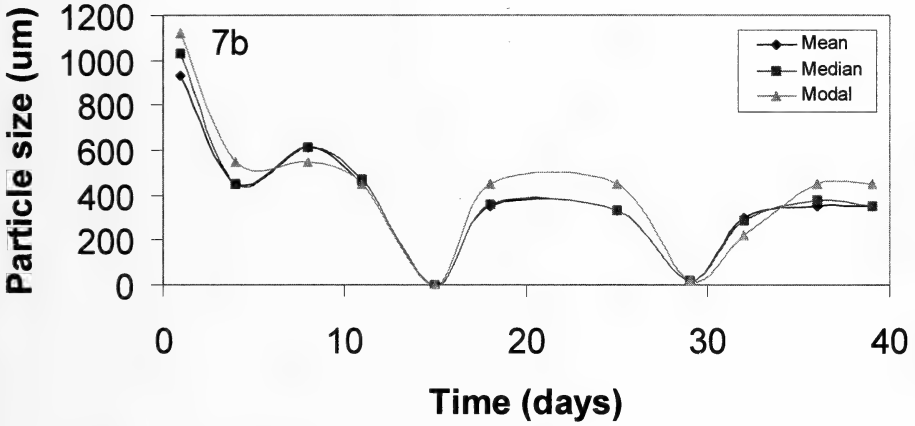
functional groups (i.e. La^{3+}-$(R\text{-}COO^{-})_3$) interact with a single lanthanide at one time, causing the structure to contract. Multiply charged cations, which are in low concentrations in the aqueous phase and are found in soils, do not disrupt aggregation of HA and appear to produce a denser aggregate.

Kinetics—The body of literature in this area relates to various aspects of kinetic studies of HS interacting with metals, industrial organics (pesticides, herbicides, etc.), radioactive species, clays, etc. (King et al., 2001; Kretzschmar and Christl, 1999; Schuessler et al., 2000; Choppin and Clark, 1991). These studies have utilized various techniques, including XPS and size exclusion chromatography, to separate and measure the chemical species interacting with HA. We used laser diffraction, a noninvasive technique, to measure the average aggregate size over a forty day period for a variety of solutions, including those at different acidities (pH 3,5,8,10), with different anions (nitrates, phosphates, carbonates), different cations (Na^{+}, Cu^{2+},

TABLE 1. CHNOS analysis of various humic structures show that percent O is typically much higher than percent N. Oxygen in HS is primarily found in carboxylate, carbonyl, and phenol groups.

	% C	% S	% N	% O	% H
Humic[59]	56.2	0.8	3.2	35.5	4.7
Fulvic[59]	45.7	1.9	2.1	41.8	5.4
Humic[60]	56.9		1.7	36.6	3.9
Soil humic[60]	54.6		3.9	35.5	5.3
Fulvic[60]	54.1		1.1	40.0	3.7
Saltwater—humic[61]	50.0		6.4	—	6.8
Freshwater—humic[61]	41.0		6.3	—	5.8
Freshwater—fulvic[61]	51.1		1.13	—	3.62
Humic[62]	48.23	1.38	3.33	42.69	4.37
Humin[62]	45.57	0.46	3.43	43.44	7.05





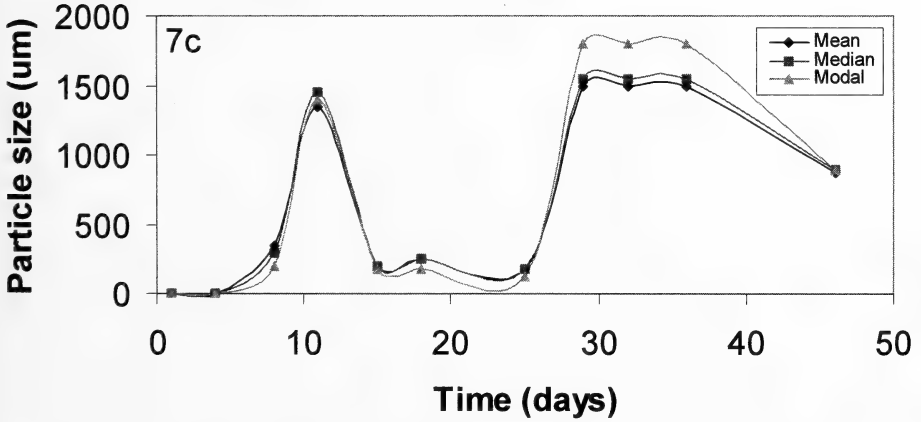


FIG. 7a. The mean, median, and modal averages of a 10 ppm HA solution at a pH of 3 was measured approximately every 4 days over a 40 day period. Data points that appear as 0 μm are typically 0.2–1.0 μm. All measurements illustrated in Figs. 7a,b,c record particle size distribution by number. Fig 7b. This is the particle size (μm) as a function of time for a 10 ppm HA solution at a pH of 7 in 0.5 M NaCl and 0.5 M KCl measured over a 40 day period. Fig. 7c. A basic HA solution at a pH of 10 measured over the same time period as in Figs. 7a,b.

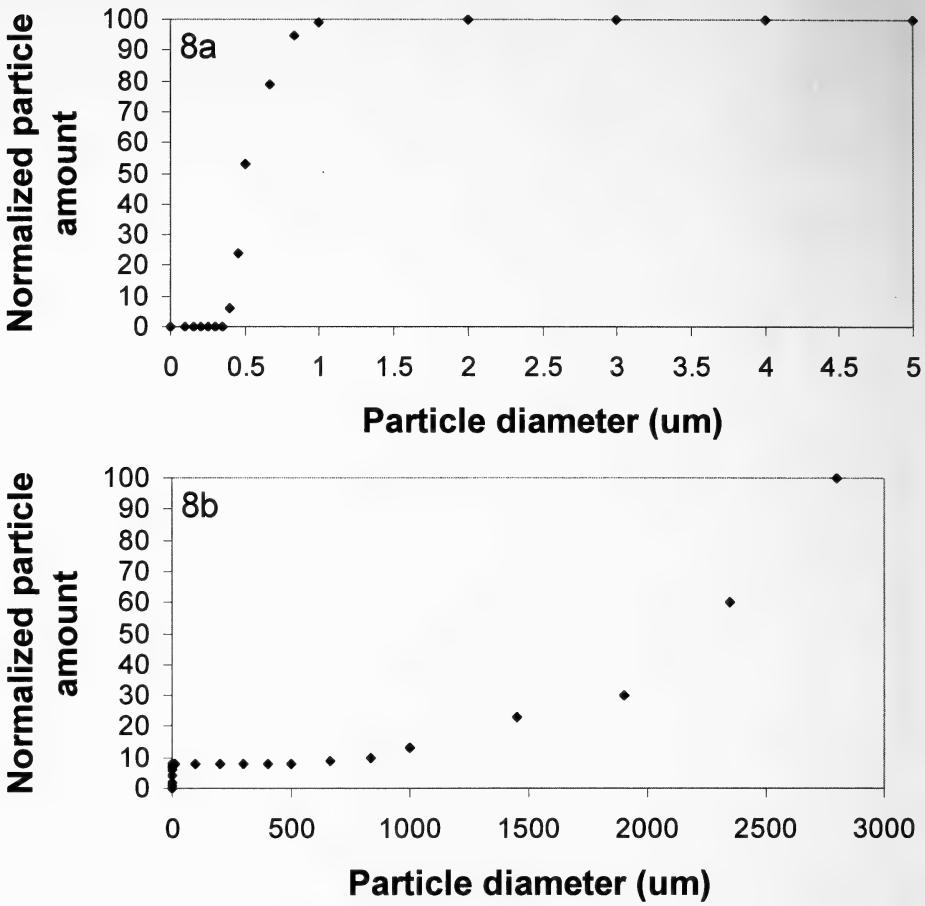


FIG. 8a. The particle size distribution when measured by the number of particles present. Fig. 8b. The particle size distribution when measured by volume. This is laser diffraction data from Day 4 (10 ppm HA) of the basic (pH = 10) solution.

La^{3+}), and different ionic strengths produced by different salt mixtures (i.e. NaCl, NaCl/KCl, NaCl/KNO_3, etc.). In this work, we hoped to better document the long-term stability of the HA aggregate under various chemical conditions. The first series of measurements were conducted twenty-four hours after the solutions were made.

Various plots (Figs. 7 a,b,c) are the results of laser diffraction measurements from HA solutions (10 ppm) at a pH of 3.0, a high ionic strength solution at a pH of 7, and at a pH of 10 for 10 ppm HA. These results demonstrate a general pattern we observed in all of our long-term measurements. Specifically, we saw periodic increases and decreases in the size of the particles in solution, but there was no reproducibility of the frequency (period) and amplitude of the measurements. In all cases, a precipitate was visible on the bottom of the individual 10 L carboys, but solutions also maintained their brownish appearance, indicating a dynamic equilibrium between the precipitate and aqueous phase. We attempted to correlate

the sinusoidal size distribution with time to some external factor (building vibrations, temperature, light exposure, transportation activity, etc.), but were unable to do so. Figures 7a,b,c illustrate the type of data that points to the aggregation and precipitation process outlined (Eqn. 1).

The largest number of particles were in the 0.5 to 0.6 μm range, with over 90% of the particles being smaller than 0.8 μm and 99.9% being smaller than 1 μm (Fig. 8a). For the same sample, although most of the particles had a diameter of 0.5 to 0.6 μm, most of the volume of HA was centralized in a few particles with diameters greater than 1000 μm (Fig. 8b). Or, a single 1000 μm particle occupies the same volume of HA as 4.6 billion 0.6 μm particles!

Choppin and Clark (1991) measured Eu^{3+} and UO_2^{+} release from HA over several days and reported a nonsymmetrical sinusoidal shaped decomposition pattern. They recorded both a weak and strong binding capacity. With our data and past work in this laboratory, an explanation for the dissociation they observed can be extended to include the aggregation processes described here. Specifically, the weak binding they described would correspond to cations being territorially trapped or the cations being held within the aggregate matrix, but not linked to a specific functional group (Hayes et al., 1995). Their strong binding would correspond to the cations site binding a specific functional group (i.e. Eu^{3+}-COO^{-}).

For the research outlined in this paper, because all solutions form a precipitate and maintain a fraction of the HA in solution, a possible mechanism is provided for the binding and transport of natural products (NP) in nature. Specifically, smaller humic aggregates bind and transport NP and larger aggregates will precipitate and accumulate a molecular fingerprint to the surrounding environment.

Conclusions—Results from our laser diffraction work demonstrate that HA, which will behave in a similar fashion to other naturally occurring organics, forms large aggregates. We show with a series of long-term kinetic studies that humics are capable of a dynamic equilibrium in which smaller (0.1-0.5 μm) and larger (3–1000 μm) aggregates are in a cycle of aggregation, precipitation, and resolubilization. We also show, using HA, that various changes in the chemical matrix (cation binding, variations in ionic strength, and acidity) can change the size of the aggregates present, but do not deter the aggregation process.

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COMPARISON OF SPECTROPHOTOMETRIC AND HPLC ESTIMATIONS OF CHLOROPHYLLS-*a*, -*b*, -*c* AND PHEOPIGMENTS IN FLORIDA BAY SESTON

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Abstract: *HPLC-derived data on the chlorophylls (*-a, -b ,-c_1/-c_2*) and pheopigments were compared to those derived from the spectrophotometric analysis of Florida Bay seston. This comparison was prompted by the rather wide spread in data from a 1996 seven-laboratory inter-laboratory comparison of chlorophyll determination in Florida Bay samples. 244 water samples from north-central and western Florida Bay phytoplankton collected during monthly sampling events (09/00–06/02) were analyzed. The spectrophotometric determination of chlorophyll-*a *(CHL*a*), using 5 separate published equations and 1 commercial data manipulation program (ChlCalc™), gave quite acceptable results (*$y = 0.9169–1.0914X$*; *$R^2 = 0.9361–0.9987$*) for CHL*a*, as compared to the HPLC-PDA (X) data. The determination of pheopigments with the commercial program gave better results (*$y = 1.0631X$*, *$R^2 = 0.463$*) than the classic determination using Lorenzen's (1967) equation (*$y = 11.178X$*, *$R^2 = 0.0271$*), but it too proved inadequate determining community "health" (*viz. *senescence, predation, resuspension). Comparisons of the determination of the chlorophylls* -b- *or* –c_1/-c_2 *by spectrophotometry versus HPLC-derived data showed that such measures were highly inaccurate, as* R^2 *values were close to zero (–0.16 to 0.04) and the slope ("m" in* $y = mX$*) gave overestimations of 1.8–5.6. It is concluded that valid CHL*a *estimates can indeed be made using spectrophotometric measures on 90% acetone extracts of Florida Bay seston (Whatman GF/F filters). However, using such polychromatic equations, it is also concluded that no meaningful estimates of pheopigments or alternate chlorophylls (*-b, -c_1/-c_2*) are possible using these methods on Florida Bay bioseston.*

Key Words: HPLC, high performance liquid chromatography, spectrophotometry, Florida Bay, phytoplankton, seston, algal blooms

Measurements of chlorophyll-*a* (CHL*a*), as well as other photosynthetic pigments, in the waters entering and within Florida Bay are integral to monitoring changes which may accompany the replumbing of the Everglades as the Comprehensive Everglades Restoration Plan (CERP; U.S. ACE and SFWMD, 1999) is enacted. In 1996, the senior author took part in a 7 laboratory-10 method interlaboratory CHL*a* determination using both unialgal cultures (3) and natural field samples (3) from Florida Bay. The interlaboratory comparison was hosted by Dr. W. L. Kruczynski of the US-EPA and the Interagency Florida Bay Program Management Committee. Results of that study revealed a wide range of results. That is, the mean of spectrophotometric and fluorometric measures was about twice

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the values obtained by RP-HPLC / PDA and, more troubling, the range in the results from analyses of natural samples often covered an order of magnitude (*e.g.* Range = 2.5 to 26.5, mean = 18.3, SD = 7.4). Analyses with pure cultures were more closely matched (*e.g.* Range = 7.7–19.9, mean = 11.8, SD = 3.7), though certainly not adequate. There are tremendous amounts of suspended carbonate marl and accompanying resuspended organics, including chlorophyll-*a* and its derivatives in Florida Bay waters (Louda et al., 2000 and references given) and it was hypothesized that perhaps the marl environment and recycled chlorophyll (*viz.* derivatives) in some way skewed these data(??). Thus, once the author's HPLC study (Louda, 2002, 2003) of pigment-based chemotaxonomy began, it was decided to collect all pertinent spectrophotometric data from samples being collected within Florida Bay. Unfortunately, a routine filter fluorometer was not available and coincident fluorometric data are lacking. However, it can be noted that, in the inter-laboratory comparison, a fluorimetric method using a 5nm bandpass (i.e. 'Welschmeyer method'. Refs. in Jeffrey et al., 1997; cf. Boyer et al., 1999) without the need for acidification gave the results closest to those we obtained in 1996 with HPLC.

The most thorough and authoritative treatment covering pigment analyses is that of Jeffrey and co-workers (1997). This volume, *Phytoplankton Pigments in Oceanography*, also applicable to fresh waters, contains 17 chapters, a compendium of identification data, and 13 appendices. This publication was the result of an immense pigment project (WG78) under the auspices of SCOR-UNESCO. One chapter, "Comparison between spectrophotometric, fluorometric and HPLC methods for chlorophyll analysis" by Mantoura and colleagues (1997) is highly pertinent to the present study.

To date, a comparison of spectrophotometric and HPLC chlorophyll analyses in a high carbonate marl—highly turbid estuary has yet to appear. Given that we were collecting monthly phytoplankton samples from the north-central western portions of Florida Bay over a 2 year span (Aug. 2000–Aug. 2002), we performed the present study in concert with our chemotaxonomic studies (Louda, 2002, 2003). While this study is not entirely novel, we feel that such investigation is requisite to the study of Florida Bay and its environs during the course of Everglades restoration. A great many determinations of CHL*a*, as a proxy for nutrient-linked phytoplankton productivity, are performed throughout Florida Bay (FIU-SERC, 2004; Louda, 2002; NOAA-AOML, 2004) and water sampling is also performed through a network of volunteers (Florida Bay Watch). While the majority of these samples are analyzed by spectrofluorometry at the Florida International University Southeast Environmental Research Center (FIU-SERC) in Miami, there is an opportunity for many more analyses to be performed using the much less expensive methodology of spectrophotometery (colorimetry). The present study then serves as 'ground truthing' for the determination of CHL*a* in Florida Bay waters using classic spectrophotometric polychromatic equations. Further, the severe limitations of those methods as applied to other chlorophylls (-*b*, -*c*) and pheopigments are detailed.

Materials and Methods—Samples were collected once per month from 18 sites (see Louda, 2002, 2003) in north-central and western Florida Bay. Water was collected in 2-L brown polyethylene bottles,

kept in the shade at collection temperature and transported to shore where they were immediately (< 4hrs. collection to freezing) filtered (Whatman GF/F) under subdued light and flash frozen in liquid nitrogen. Storage and transport of the aluminum foil-wrapped twice-folded and blotted filters was on dry-ice. Pigment extraction and analyses occurred with 2 weeks of collection.

Pigments were extracted using exactly 3.0 mL of 90% aqueous acetone containing a known amount of copper mesoporphyrin-IX dimethyl ester (Cu-Meso-IX-DME) as an internal standard (= IS). Extraction occurred with grinding in a pre-chilled (*viz.* frozen) modified Potter-Elvehjem tissue homogenizer (Kontes™ 8886000 series), sonication, and steeping for 1-2 hours in a refrigerator. The extraction mix was centrifuged, decanted and the moist filter paper pellet was recentrifuged in a centrifugal filter device (Amicon Ultrafree-CL™), giving a total extract recovery of 93+% (2.8/3.0 mL). The pooled raw extract was then filtered through a 0.45 μm syringe filter. All procedures were at ice bath (~ 0–2°C) temperatures. It must be noted that modification of standard tissue homogenizers (rounded pestle) by slicing off pieces of the tip to form an irregular pointed tip, greatly enhanced the complete disruption of the GF/F filter and seston. This, with sporadic sonication (homogenizer mortar immersed into bath style sonicator), caused the extraction mix to become quite homogeneous and without any remaining identifiable filter pieces. Prolonged steeping (24+hrs) brought out only minor amounts of additional pigment, 2–5% as a maximum, when compared with identical samples steeped for only 2 hours. Potential alteration (*viz.* oxidation, isomerization) of pigments by letting them remain in solvent does not warrant the small additional yield. It must be noted that this study utilized only 90% aqueous acetone as an extractant and was not designed to investigate alternate extractants (cf. Wright et al., 1997). Dimethylformamide (DMF) is reported to be superior for certain recalcitrant pigments (notably CHL*b*) but it is a strong liver toxicant which is readily absorbed through the skin and is not recommended by SCOR-UNESCO for that reason (Wright et al., 1997). However, in a recent study of pigment extraction from Everglades periphyton, we found that a mixture of acetone/methanol/dimethylformamide/water (30:30:30:10, v/v/v/v) was not only an excellent extractant, based on yields per dry weight sample, but also aided in giving better (*viz.* sharper) peak shapes for the polar pigments (*i.e.* chlorophyllides, chlorophylls-*c etc.*: Hagerthey et al., 2003). A mixture of acetone/ methanol/water (45:45:10) also worked quite well and gave excellent early running peak shapes.

Exactly 1.000 mL of the raw extract was added to a pre-chilled vial containing 0.125 mL of an ion pairing solution (cf. Mantoura and Llewellyn, 1983). This mixture formed the injectate and 0.100 mL (100 μL) subsample was loaded onto the HPLC column. The HPLC conditions and gradient are given elsewhere (Louda et al., 1998, 2000, 2002). Basically, the system includes a ThermoSeparations Model 4100 quaternary LC pump, a Rheodyne injector with an 100 μL loop, a 3.9 × 150 mm Waters NovaPak (4 μm C18) reverse-phase column and a Waters Model 990 photodiode array detector. A linear solvent gradient (Louda et al., 2002), not dissimilar to the UNESCO system (Jeffrey et al., 1997) and based on that given by Mantoura and Llewellyn (1983) and Gieskes and Kraay (1983) was utilized. The HPLC system was calibrated using over 90 known chlorophylls, chlorophyll derivatives and carotenoids. Additionally, an internal standard (Cu-MESO-IX-DME) was added to the extractant such that both recovery (detection) of the internal standard (IS) and chromatographic performance could be monitored. That is, we calculated a detection conversion factor by dividing the added amount of IS by that we detected during the HPLC analysis. A system response factor was applied to all pigments based on the ratio $IS_{added}/IS_{detected}$. The correction factors ranged from 1.1–1.3x. Pigment detection and quantitation derived from the Beer-Lambert relationship using PDA data (AU^*min) and published extinction coefficients adjusted to 440 nm (chlorophylls, chlorophyllides, carotenoids), 410 nm (pheophytins, pheophorbides, pheophorbide steryl esters), or 394 nm (CuMeso-IX-DME = IS: see Louda et al., 2002).

Ultraviolet/visible (UV/Vis) spectrophotometry was performed using a Perkin-Elmer Lambda-2 instrument which was calibrated with a holmium oxide standard for both wavelength and absorptivity. The UV/Vis spectrum of an additional 1.0 mL aliquot of the filtered raw extract was recorded and instrument-derived absorption values recorded at 630, 645, 647, 663, 664, 665 and 750 nm for use in the polychromatic equations to be tested. Next 1 drop of 2% HCl (w/v) was added, the solution mixed once with a Pasteur pipette and the spectrum re-recorded, this time taking absorption at 665 and 750 nm for "pheopigment" estimations.

There is a report that the use of a reverse optic photodiode array (PDA) spectrometer will lead to 6–9% lower estimation of chlorophylls-*a*/-*b* (Latasa et al., 1996). Those authors attribute that inconsistency

to chlorophyll fluorescence contaminating the simultaneous reading by the PDA. However, a more recent study (Dunne, 1999) reports that a small depression, not at the 6–9% level, is actually due to the wider bandwidths of PDA instrument and that all regressions, conventional dispersive and PDA, yielded regressions that were significant ($p < 0.001$) with positive slopes and intercepts not significantly different than zero. These reports are covered here to reveal that spectrophotometry is a quite reliable tool but one which must be standardized (QA) and monitored (QC). The Lambda-2 instrument used here is a conventional dispersive double beam instrument with a narrow bandwidth and scans were made at 240nm/min. The PDA used herein for HPLC pigment detection was constantly monitored for sensitivity with the internal standard as well as known pigment determinations.

The so-called "simultaneous equations" were taken from the literature (see references) and, along with others not used herein, can be found in the review of Jeffrey and Welschmeyer (1997) which is Appendix F in Jeffrey and co-workers (1997). All results of the equations tested, (shown below) are in µg/mL, except Lorenzen (1967) and ChlCalc™ that give mg/m^{-3} (µg/L) directly. "A" is the absorption at the wavelength (nm) indicated by subscript:

"SCOR-UNESCO (1966)" 90% acetone;

$$\text{CHL}a = 11.64\ A_{665} - 2.16\ A_{645} + 0.10\ A_{630} \quad (1)$$

$$\text{CHL}b = -3.94\ A_{663} + 20.97\ A_{645} - 3.66\ A_{630} \quad (2)$$

$$\text{CHLs}c = -5.53\ A_{663} - 14.81\ A_{645} + 54.22\ E_{630} \quad (3)$$

Jeffrey and Humphrey (1975) {= J&H'75} 90% acetone;

$$\text{CHL}a = 11.85\ A_{664} - 1.54\ A_{647} - 0.08\ A_{630} \quad (4)$$

$$\text{CHL}b = -5.47\ A_{664} + 21.03\ A_{647} - 2.66\ A_{630} \quad (5)$$

$$\text{CHLs}c = -1.67\ A_{664} - 7.60\ A_{647} + 24.52\ A_{630} \quad (6)$$

Jeffrey and Humphrey (1975)/Humphrey (1979) {= J&H'75/H'79} 90% Acetone. (chromophyte modification)

$$\text{CHL}a = 11.47\ A_{664} - 0.40\ A_{630} \quad (7)$$

$$\text{CHLs}c = 24.36\ A_{630} - 3.73\ A_{663} \quad (8)$$

Lorenzen (1965) Chl*a* corrected for 'pheopigments'. 90% acetone.

$$\text{CHL}a = [26.73(A_{665}{}^{o} - A_{665}{}^{a})v]/V \quad (9)$$

$$\text{Pheo} = [26.73(A_{665}{}^{a} - A_{665}{}^{o})v]/V \quad (10)$$

Here $A_{665}{}^{o}$ and $A_{665}{}^{a}$ are absorption at 665nm before and after acidification, v = volume of the pigment extract, V = volume of the water filtered, and 26.73 is an absorption coefficient correction for the ratio of these pigments with pure chlorophyll.

A commercial product "Chlorophyll Calculator™ (ver. 1.11 © 1993. SoftLabWare™, as distributed by WindowChem™, Fairfield, Ca.) was also tested. The equations contained within that commercial product are unknown to the authors.

RESULTS AND DISCUSSION—A total of 100 to 2,000 mL of sample, depending upon turbidity, of Florida Bay water was able to be filtered. The carbonate marl load of these waters impeded filtration and forms one of the reasons for this study. That is, does the presence of fine-grained carbonates and their inherent basicity affect pigment extracts?

Spectra of the extracts gave A_{664} values between 0.008 and 0.250, with a majority between 0.05 and 0.15. No attempt was made to sort results by the absorbance of the crude extract and no relation was apparent upon causal examination.

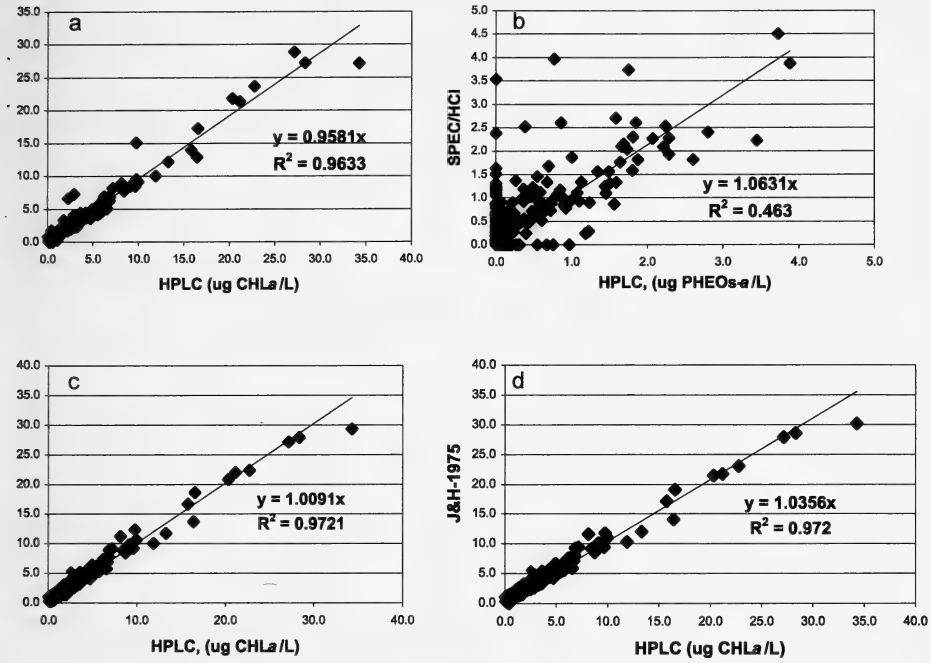

FIG. 1. (a, c, d) Determination of CHL*a* by HPLC (x-axis) v. the methods of (a) ChlCalc™, (c) SCOR-UNESCO, 1966, and (d) Jeffrey and Humphrey, 1975. (b) determination of "pheopigments" by HPLC (x-axis) v. ChlCalc™.

Some 244 samples, collected between September 2000 and May 2002, are included in this report.

Figures 1a, 1c, 1d, 2a and 3a are plots of the HPLC-determined chlorophyll-*a* ($\sum$ CHL*a* = CHL*a* + CHL*a*-epimer + CHL*a*-allomer + CHLide-*a* + pyroCHLide-*a*: namely, all CHL*a* chromophoric species, x-axis) as a function of CHL*a* determined by the indicated spectrophotometric methods. All of the correlations yielded an approximate 1:1 relationship (slope ~ 1.0) with r-squared values [R^2] close to unity. All of the CHL*a* spectrophotometric estimations trace their origins to the works of Arnon (1949), Richards and Thompson (1952), and the revision of Parsons and Strickland (1963). Only slight alterations in the coefficients used have occurred since then. Therefore, the finding that all of these estimates are very close is not too surprising. All correlations were forced through the origin (0,0), as needs to be done to maintain Beer-Lambert constraints. Resulting slopes and r-squared values (R^2) for these comparisons are given in Table 1. Even though the estimation of CHL*a* 'chromophores' was acceptable, the inability of these methods to detect the altered chlorophylls-*a*, such as chlorophyllide-*a* or chlorophyll-*a*—allomer, does not allow any inference as to community health (*e.g.* senescence).

The estimation of the pheopigments, a term which SCOR-UNESCO WG78 does not approve of, but acknowledges due to its widespread use in the literature (Jeffrey and Welschmeyer, 1997), includes measuring all of the pigments with

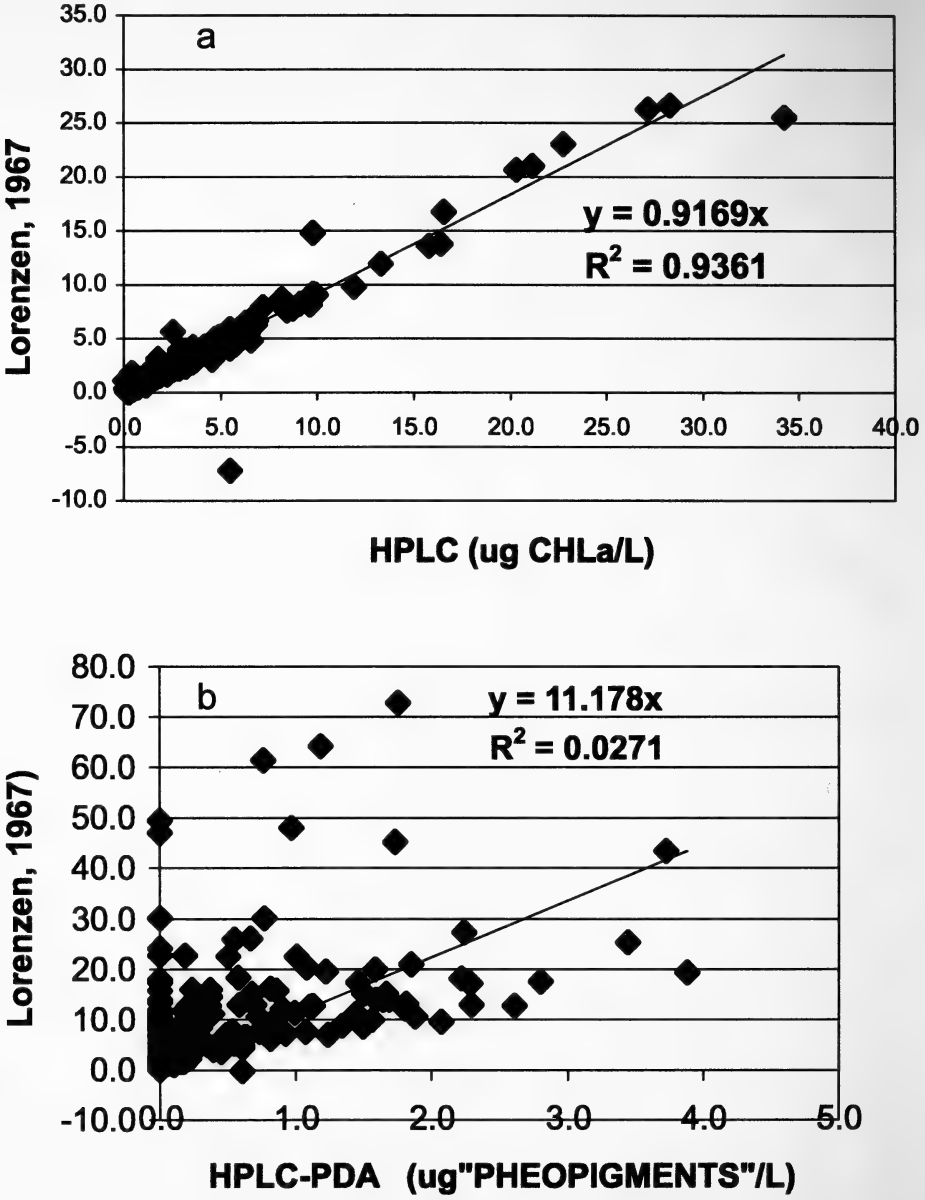

FIG. 2. The 'acidification' method of Lorenzen, 1967. (a) Determination of CHL*a* v. HPLC(X-axis). (b) Determination of "pheopigments" v. HPLC (x-axis).

a pheophorbide-*a*-like (PHide*a*) chromophore. This measure, once verified, can give important information as to the 'health' of a community, predation and/or to the amount of recycled/resuspended material in the seston (Louda et al., 1998, 2002; Millie et al., 1993). However, a rapid and facile method, either by spectrophotometry or fluorometry, is apparently still lacking (see the caveats reviewed by Jeffrey

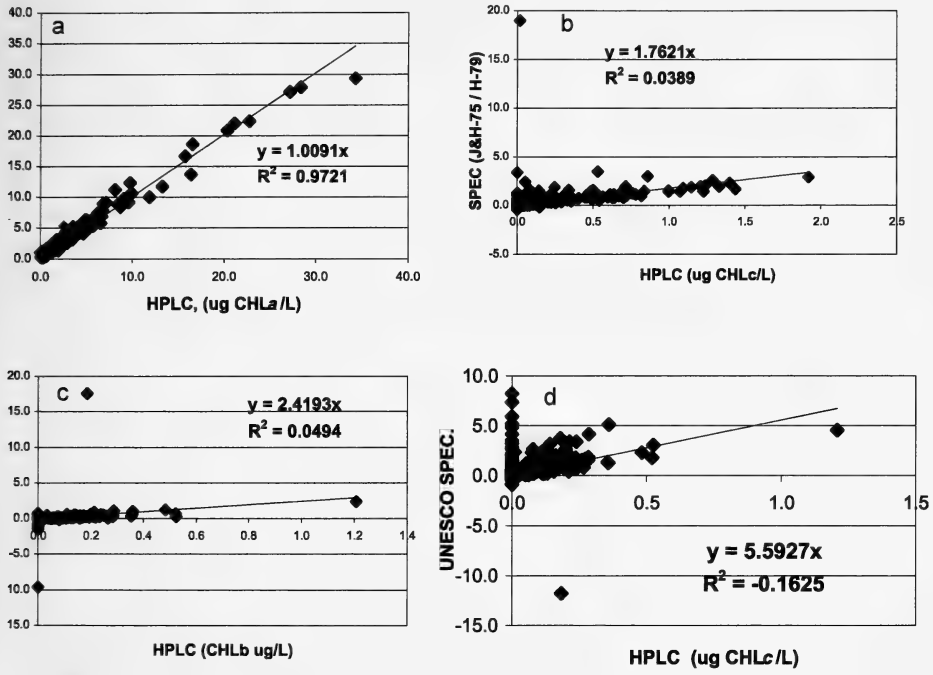

FIG. 3. (a) CHL*a* determined by HPLC (x-axis) v. the method of Jeffrey and Humphrey, 1975 with Humphrey, 1979 modification. (b) CHL*b* determined by HPLC (x-axis) v. the method of SCOR-UNESCO, 1966. (c-d) Determination of Chlorophylls-*c* by HPLC (x-axis) v. (c) the method of Jeffrey and Humphrey, 1975 with Humphrey, 1979 modification. and (d) SCOR-UNESCO, 1966.

and Welschmeyer, 1997). In the present case, we examined the spectrophotometric estimation of pheopigments in Florida Bay seston by the acidification method using the commercial ChlCalc™ software (Fig. 1b) and the method of Lorenzen (1967: Fig. 2b). The method of Lorenzen (1967) gave a slope of about 11 and essentially no correlation ($R^2 = 0.0271$). However, even though the correlation of the commercial (ChlCalc™) software was poor ($R^2 = 0.463$), the slope was close to unity ($y = 1.0631x$). It must also be pointed out that, in this relationship (Fig. 1b), a considerable number of samples either had pheopigments and were not estimated, or they were estimated and were not present. The only conclusion possible is that, if information on pheopigments is required, then HPLC methodology must be utilized. This is especially true if information which details predation (*viz.* pyro-pheophorbide-*a*), senescence (*viz.* pheophytin-*a*), or sediment resuspension (both) is required. Details of these processes can be found in reviews by the senior author (Baker and Louda, 1986, 2002). In the present study, the value [pheopigments] determined by HPLC was the sum of pheophorbide-*a* (PHide*a*), PHidea-allomer, pyro-PHide*a*, pheophytin-*a* (PHtin*a*), PHtin-*a*-epimer, PHtin-*a*-allomer, pyro-PHtin-*a*, PHide*a*-steryl esters, and pyroPHide*a*-steryl esters (see Louda et al., 2000).

TABLE 1. Compiled regression data (slope and R^2) for the comparison of chlorophylls and pheopigments in Florida Bay seston determined by HPLC (x) versus spectrophotometric (y) methodologies.

Pigment estimated Method (y)	Slope[a]	r – Squared value[a] [R^2]
Chlorophyll-a		
ChlCalc™ software	0.9581	0.9633
SCOR-UNESCO, 1966	1.0091	0.9721
Jeffrey & Humphrey, 1975	1.0356	0.9720
Lorenzen, 1967	0.9169	0.9361
J&H'75/Humphrey, 1979	1.0332	0.9710
Pheopigments (a)		
ChlCalc™ software	1.0631	0.4630
Lorenzen, 1967	11.1780	0.0271
Chlorophyll-b:		
SCOR-UNESCO, 1966	2.4193	0.0494
Chlorophyll-c:		
SCOR-UNESCO, 1966	5.5927	–0.1625
J&H'75 with Humphrey, 1979	1.762	0.0389

[a] Regression data (slope and correlation coefficient, R^2) determined using Microsoft Excel.

We also compared HPLC determinations of chlorophyll-*b* (CHL*b*: Figure3b) and the chlorophylls-*c* (=$\sum$ CHLc_1 + *CHL*c_2 : Figs. 3c and 3d). In these cases, approximately 1.8 to 5.6 overestimations with no correlation were found. Again, if information on the presence and abundance of the accessory chlorophylls (-*b*, -*c*) is required, then only HPLC data will suffice.

Lastly, we considered the amount of material required for a reasonable spectrophotometric CHL*a* estimate. That is, Jeffrey and Welschmeyer (1997) state: "Ideally, enough seawater should be filtered to yield an absorbance (optical density) >0.1 at 664 nm when using the spectrophotometric acidification technique."

Examination of the rank-ordered distribution of raw extract absorption values (Fig. 4) obtained during the present study reveals that only 50 (20.5%) of the 244 samples analyzed met the above criterion. The slopes and correlation between the spectrophotometric estimates and the HPLC-derived data (Table 1) indicate that these estimates were quite acceptable (*viz.* R^2 values = 0.93 –0.97 with slopes = 0.92 – 1.03), regardless of the absolute value of the absorption of the extract.

Visual examination of the raw data revealed that there likely was a lower degree of precision between the spectrophotometric estimations and the HPLC-determined values when A_{664} of the raw extract was below about 0.02AU. Indeed, consideration of the 20 (8.2%) samples, out of the 244, with $A_{664} < 0.02$AU revealed poorer correlation coefficients (Lorenzen, 1975/$R^2 = 0.6284$; SCOR-UNESCO 1966/$R^2 = 0.7957$; Jeffrey and Humphrey, 1975/$R^2 = 0.778$). However, slopes (1.0256, 1.0468, 1.0767, respectively) were still nominally at unity (3–7% overestimations). Obviously, calculation of RSDs and similar indices would allow discarding of true outliers. However, comparison of the spectrophotometric techniques with HPLC data requires inclusion of all data. That is, if only the spectrophotometric data were

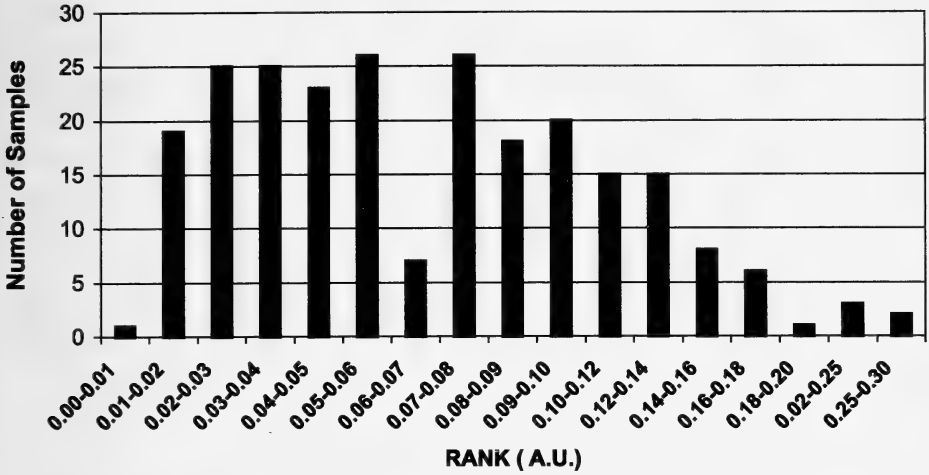

FIG. 4. Rank ordered distribution of absorbance values (A.U. at $\lambda = 664$ nm) for the 244 samples of northern Florida Bay seston included in this study.

available, a result would not be detectable as an outlier and would be included in any data set. Replicate runs on our HPLC system, using the same or different extracts of the same sample, reveal very small (2–5%) variations (Louda unpubl. data; cf. Winfree et al., 1997).

The comparisons made during this study were derived from water samples containing from 0.07 to 34.27 μg/L. One sample from an isolated water body well within the mangrove transition zone (Mrazek Lake, S = 9 psu) gave a total CHL-*a* value of 441 μg/L and was left out of the calculations reported here. However, inclusion of the Mrazek Lake data changed the CHL*a* regressions very little (*e.g.*, ChlCalc™ slope = 1.0914, $R^2 = 0.9987$) but severely skewed the pheopigments calculations (*e.g.*, ChlCalc™ slope = 0.4166, $R^2 = -0.2109$) due to the large 'lever arm' imparted by that single sample.

The rank-ordered distribution of CHL*a* concentration in the Florida Bay water samples investigated during this study is given as Figure 5. Evaluation of the pigment-based chemotaxonomy on these samples utilized zeaxanthin/echinenone, chlorophyll-b/lutein, fucoxanthin, peridinin and alloxanthin/α-carotene as biomarkers for cyanobacteria, chlorophytes, diatoms (chrysophytes), dinoflagellates and cryptophytes, respectively (see Louda, 2002, 2003). Simply put, the low (0.1–2.0 μg/L) values derived from (diatom) non-bloom sequences in the north central bay, the moderate values (2–6 μg/L) came mainly from mixed phytoplankton communities (diatom, dinoflagellate, cryptophyte, chlorophyte) of the western bay, and the high values (6–35 μg/L) were associated with cyanobacterial bloom sequences in the north-central bay.

DISCLAIMER—Mention of trade names in text does not constitute an endorsement by the authors or their funding agencies (DOC, NOAA, NMFS,

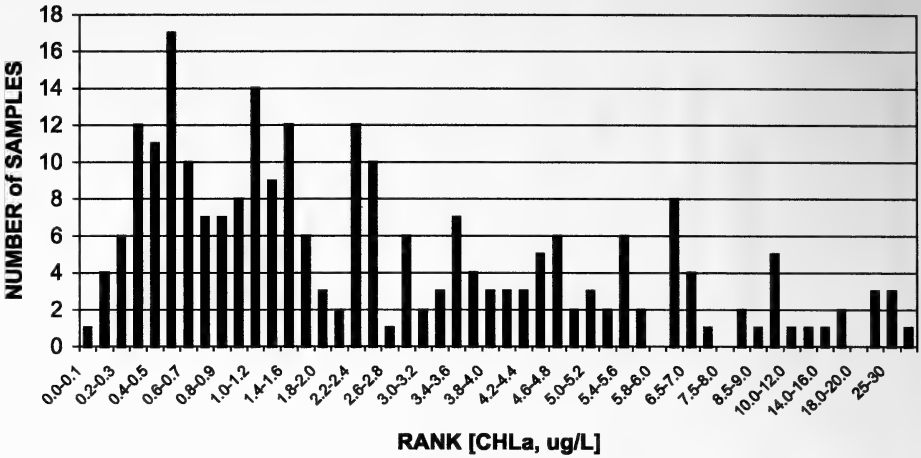

FIG. 5. Rank ordered distribution of CHL*a* concentration (μg/L) in the 244 samples of north-central and western Florida Bay water analyzed from September 2000 through May 2002.

SFERPM). Rather, trade names were cited only to indicate a style or level of quality. Alternate suppliers for each item are available and should suffice.

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RATES OF NATURAL HERBIVORY AND EFFECT OF SIMULATED HERBIVORY ON PLANT PERFORMANCE OF A NATIVE AND NON-NATIVE *ARDISIA* SPECIES

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ABSTRACT: *Non-native species may have a higher fitness in their adventive range relative to their native ranges due to release from enemy pressure. The enemy release hypothesis implies that introduced plant species should suffer lower levels of herbivory than native congeners. The goal of this study was to quantify rates of herbivory on the southern Florida invasive shrub* Ardisia elliptica *and its native congener* A. escallonioides *and evaluate how different rates of leaf area loss may affect the growth and potential reproductive output of each species. Consistent with the enemy release hypothesis,* A. elliptica *experienced significantly less herbivore damage than* A. escallonioides. *Although there was a weak and generally non-significant response to simulated herbivory,* A. escallonioides *was consistently more negatively impacted by leaf area loss. During this study, two folivores and one seed borer were observed feeding on* A. elliptica.

Key Words: Ardisia elliptica, Ardisia escallonioides, enemy release hypothesis, invasive non-native species, simulated herbivory.

IT has been hypothesized that non-native species may have higher fitness in their adventive range relative to their native range because they have been released from herbivore (enemy) pressure (Elton, 1958; Crawley, 1987; Blossey and Notzold, 1995). Herbivory (*sensu latu*; e.g., foliar, stem, seed, sap, root, etc.) reduces plant fitness directly through the loss of valuable tissues (Fox and Morrow, 1983; Marquis, 1992; Byington et al., 1994) and indirectly through plant allocation of resources to defense. Although there are numerous mechanisms through which non-native species may become demographically successful in their new ranges, release from enemy pressure can only explain the success of an invasive species if the species experiences reduced regulation by plant enemies in its adventive range relative to its native range. In some cases, improved plant performance represents a plastic phenotypic response of the invading species to a relatively benign environment. However, in other cases, improved performance may have resulted from the evolution of novel genotypes that are relatively more vigorous but poorly defended (the Evolution of Improved Competitive Ability hypothesis of Blossey and Notzold, 1995; see also Willis and Blossey, 1999; Willis et al., 1999; Willis et al., 2000).

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Regardless of the mechanism by which non-native species experience improved plant performance, increases in growth, survival or reproduction gained by non-native species may give them a competitive advantage over natives that are being suppressed by native enemies (The Enemy Release hypothesis; see Keane and Crawley, 2002). Three assumptions of the enemy release hypothesis are: 1) specialist enemies of the non-native species are not introduced to the new range; 2) host switching of specialist enemies from native congeners is minimal; and 3) generalist enemies have a stronger effect on native competitors. Unfortunately, this hypothesis has not been explicitly tested through direct comparisons of the effect of herbivory on population dynamics between populations of invasive species in their native and adventive ranges (Keane and Crawley, 2002).

Evidence for the ability of herbivory to control populations of invasive non-native species can be found in the biological control literature (see reviews by Center et al., 1997; Simberloff et al., 1997). For a biological control agent to be effective at controlling invasive species, it needs to damage its host enough so that individual plant growth, survival, or reproduction is decreased, thereby decreasing the competitive ability of the host. However, even if herbivory significantly reduces individual plant performance, it may not affect population growth if it targets life history transitions or life history stages that have low sensitivities and elasticities (*sensu* Caswell, 2001). Recent studies have recommended the use of projection matrix models to evaluate the potential impact of biocontrol agents on the population dynamics of invasive species (Shea and Kelly, 1998; McEvoy and Coombs, 1999).

There has been some debate over the use of non-native species to control invasive species because in some instances biocontrol agents have also attacked native species, causing local extinctions, affecting food web dynamics and changing community structure (Simberloff and Stiling, 1996). Identification and development of native biocontrol agents may be a feasible alternative where use of non-indigenous control agents is deemed risky. There are some instances where herbivores and pathogens of native congeners have been known to switch hosts and control populations of invasive species (Creed and Sheldon, 1995). Thus in the development of biological control programs, populations of invasive species need to be examined carefully for potentially useful native enemies.

In southern Florida, *Ardisia elliptica* Thunberg (Myrsinaceae) is a non-native, tropical understory shrub that invades both disturbed and undisturbed upland habitats. It is native to southeast Asia and invasive in southern Florida, the Caribbean, Cook Islands, French Polynesia and Hawaii (Langeland and Burks, 1998; Space and Flynn, 2002). It readily excludes native species and forms dense monospecific stands (Seavey and Seavey, 1993; Langeland and Burks, 1998). It was imported by 1900 as an ornamental (Gordon and Thomas, 1997) and since then has spread throughout the region. It invades moist undisturbed forests and disturbed habitats, forming dense thickets that exclude native species (up to 350 plants m^{-2}; Koop, 2003). Most populations suffer very little herbivore damage, although some plants may suffer moderate amounts of herbivory (see below). For additional information on the natural history of *A. elliptica* see Koop (2004).

The main goal of this study was to examine how herbivory influences fitness components of the non-native *A. elliptica* and the native *A. escallonioides* Schiede & Deppe ex Schldl. & Cham., a tropical understory congener in southern Florida. The specific objectives were to 1) measure population herbivory levels for these species; and 2) evaluate how loss of leaf area due to simulated herbivory affects plant size and potential reproductive output after one year. Casual observations made of native herbivores feeding on *A. elliptica* are also reported.

METHODS—*Natural herbivory*—To estimate current rates of herbivory on both native and non-native *Ardisia* species, natural areas containing both species were sampled. Seven parks and reserves within Miami-Dade and Broward Counties in southern Florida were found to contain both species. In three of these parks (Matheson Hammock, Everglades National Park, Secret Woods), populations of the two species were sympatric, while in the other four parks (Deering Estate, Trade Winds, Fern Forest, Owaisa Bauer) the species were separated by approximately 0.1 km to 1.0 km. At all sites, *A. escallonioides* was studied within tropical hardwood forests. Where *A. elliptica* was sympatric with *A. escallonioides*, it was also studied within hardwood forests, otherwise, it was studied in disturbed sites that were dominated by a mixture of native and non-native vegetation.

At each site and for each species, 20 plants were haphazardly selected along and off trails. A minimum distance of five meters between plants was maintained except where populations were small and densely spaced. From each plant, 13-15 leaves were randomly selected from high, medium and low-hanging branches and placed within separate envelopes. At the University of Miami, leaves were scanned using an Epson Perfection 1640SU scanner and analyzed with the software WINFOLIA (v. 5.0a, Regent Instruments Inc.). With an interactive interface, WINFOLIA estimates total leaf area present and leaf area missing ("holes"). In situations where leaf area was missing along the margins of leaves, the original leaf margin was drawn digitally to estimate leaf area missing. Mean percent herbivore damage to leaves for each plant was estimated. Differences in percent herbivory between the species of *Ardisia* at each site were analyzed using a Wilcoxon two-sample test (Proc Npar1way; SAS v. 8.0). Mean rank scores were used for tied variates. Probabilities reported are based on the two-sided, *t*-approximation.

Simulated herbivory—Although simulated herbivory does not completely mimic natural herbivory (Smith, 1989), it can yield unbiased estimates of the potential effect of herbivory on plant fitness (Tiffin and Inouye, 2000) and is logistically easier to conduct. As a preliminary approach to examining the effect of herbivory on plant size and reproductive output, a simulated herbivory experiment was conducted under shadehouse conditions. Seeds of *Ardisia elliptica* and *A. escallonioides* were germinated in flats. Plants were inoculated with soil mychorrizae by laying flats of seedlings on a layer of chopped roots obtained locally. Seedlings were transplanted to 1-gallon pots in June 2000 using a potting soil mixture composed of Canadian peat moss, fine pine-bark chips, perlite and local peat (5, 4, 3 and 1 parts each, respectively). Plants were given a liquid fertilizer and a slow-release granular fertilizer (Osmocote) at planting. They were watered every two or three days, or as needed. Plants were maintained in a shadehouse facility near the University of Miami and were grown under 65% shade cloth.

After allowing plants approximately six months to establish in pots, they were randomly assigned to one of ten levels of simulated herbivory (0, 3, 6, 9, 12, 15, 18, 21, 24 or 27%; two replicates per treatment). Starting in September 2000 for *Ardisia elliptica* and in January 2001 for *A. escallonioides*, all initial leaves were subjected to treatment. Every two to three weeks following that, all new mature leaves were also subjected to treatment, until November 2001 when the experiment was terminated. To determine the appropriate amount of leaf area to remove, the length and maximum width of each leaf (perpendicular to the midrib) was used to estimate total leaf area using an allometric model developed for each species (*A. elliptica*: LA (mm^2) = 0.6557 × L (mm) × W (mm), R^2 = 0.99, N = 171; *A. escallonioides*: LA (mm^2) = 0.6718 × L (mm) × W (mm), R^2 = 0.99, N = 158). Given that the area removed by a standard single-hole hole-puncher is approximately 30 mm^2, an appropriate number of holes were punched out of each leaf. Holes were punched adjacent to each other along leaf margins and then inward toward the midrib. A somewhat continuous range of herbivory rates was chosen to be able to

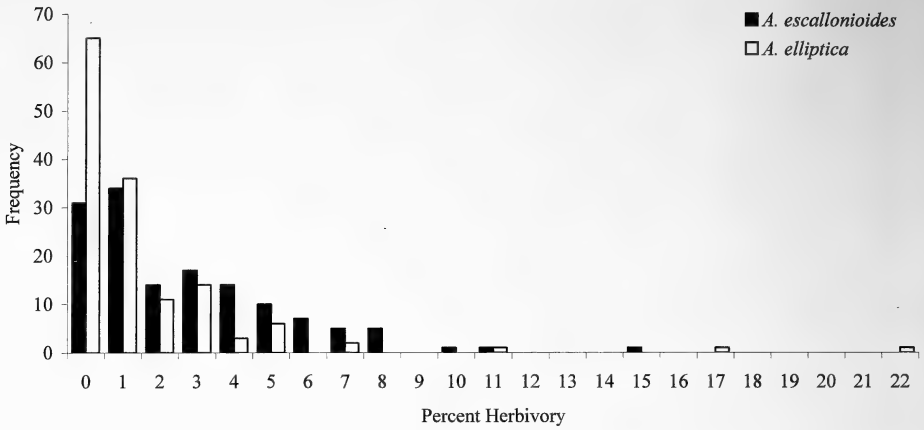

FIG. 1. Distribution of plant herbivory rates (percentage leaf area missing) for *A. elliptica* and *A. escallonioides*. Data were pooled for all populations and represent an N of 140 for each species.

detect plant response to a range of different rates of leaf loss. Furthermore, this experimental design allowed use of a regression analysis approach, which is more powerful at detecting subtle changes among treatment groups than an analysis of variance (Pedhazur, 1997). Throughout the one year experimental period, both *Ardisia* species grew continuously.

Initially and then every two months, plant basal diameter and height were recorded as estimates of plant growth. For *A. elliptica* and *A. escallonioides*, number of branches and stem tips, respectively, were recorded as estimates of potential reproductive output. A demographic study of *A. elliptica* showed that number of branches is a better predictor of fruit production than either stem basal diameter or plant height (Koop, 2003). It was assumed that the number of stem tips would be the best predictor of fruit production for *A. escallonioides* since this species bears its fruits in terminal clusters at the ends of stems (Pascarella, 1998).

Final plant basal diameter, height and number of branches / stems in November 2001 were analyzed using simple regression analysis with herbivory level as the independent variable (Proc REG, SAS v. 8.0). Despite random assignment, treatment was positively associated with initial height of *A. elliptica* ($r = 0.44$; $p = 0.0498$; $N = 20$). To control for this anomaly and to control for the effect of initial plant size on plant response variables, initial basal diameter or height was used as a covariate in the regression analyses of both species. Initial basal diameter was used in the analysis of final basal diameter, while initial height was used in the analysis of final height and number of branches/stems. Partial correlation coefficients of the effect treatment were reported since they represent the effect of treatment after both the independent and dependent variables have been residualized on the covariate. To determine whether species responded differently to leaf area loss, standardized regression coefficients were compared using a t-test (Howell, 1997). Although, it may be unrealistic for this study to predict the effect of long-term sustained herbivory on plant performance from only a one year treatment, a regression approach should allow identification of early trends.

RESULTS—*Field assay of herbivore damage*—Estimates of mean percent herbivore damage to leaves for each plant ranged between 0% and 22% for the non-native *Ardisia elliptica* (mean = 1.51%; N = 140 plants; SE = 0.24; Fig. 1) and between 0% and 15% for the native *A. escallonioides* (mean = 2.69%; N = 140 plants; SE = 0.22; Fig. 1). *A. elliptica* suffered significantly less herbivore damage than *A. escallonioides* at five of the seven sites (Wilcoxon tests; Fig. 2). At the two remaining sites (Matheson Hammock and Fern Forest), herbivore damage was not significantly different. The relatively high rate of damage for the non-native observed at Fern Forest (2.15%, N = 20 plants, SE = 1.10) was due to particularly

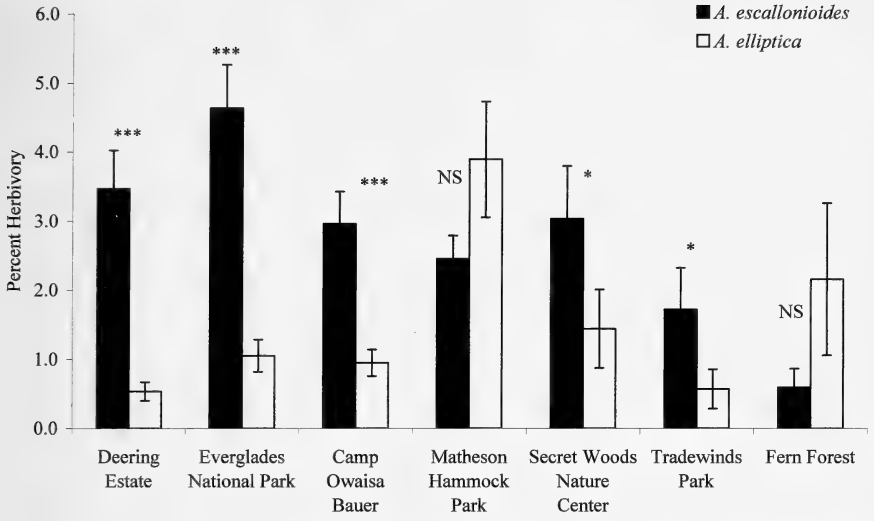

FIG. 2. Foliar herbivory rates (percentage leaf area missing) on the invasive non-native *A. elliptica* and the native *A. escallonioides* across seven sites in southeastern Florida. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS = Not Significant. Error bars represent one standard error.

heavy damage to one plant (22% of leaf area removed; see Fig. 1). Omitting this individual resulted in a mean of 1.10% damage (N = 19 plants, SE = 0.33).

Shadehouse study of effects of herbivory—Simulated herbivory of up to 27% of leaf area had no significant effect on growth or potential reproduction of the non-native species over the one-year experimental period. For the native, simulated herbivory had no effect on basal diameter or potential reproduction, however, it did have a significant negative effect on plant height ($t = -2.16$, $p = 0.0451$, partial $R^2 = 0.22$; Table 1). Even though treatment had little to no effect on plant performance of either species, it had a stronger effect on the native *A. escallonioides* than on the non-native *A. elliptica*. Examination of the standardized regression coefficients demonstrates that treatment consistently had a stronger negative effect on the native (Table 1). Partial R^2 values of treatment also show that treatment explained a greater proportion of the variation in growth and potential reproduction of the native Ardisia than of the invasive *Ardisia* (Table 1). However, despite this consistently more negative effect of treatment on the native species, only the difference in response of height was significantly different between species ($t = 2.35$; $P < 0.05$; Fig. 3).

Incidental observations of herbivores—During this study and several other studies of *A. elliptica* (Koop, 2003), several herbivorous insects were observed feeding on *A. elliptica*. In a hardwood forest in Everglades National Park, a saddle back caterpillar (*Sibine stimulea*) was seen feeding on the leaves of *A. elliptica*. The same species attacked several plants of both species of *Ardisia* during a shadehouse study. The caterpillars damaged leaves extensively, often removing over 90% of leaf

TABLE 1. Effect of simulated herbivory on final plant size of *A. elliptica* (X) and *A. escallonioides* (N) after controlling for initial plant basal diameter or height (see text). Shown are the unstandardized and standardized regression coefficients, test statistics and the R^2 value of the overall model. Partial R^2 values are based on Type II sums of squares.

	Regression Coefficients							
	Unstandardized			Standardized	Treatment Effect			
	Intercept	Control	Treatment	Treatment	t	p	Partial R^2	Model R^2
Basal Diameter								
X	1.1653	−0.3304	−0.0005	−0.0446	−0.18	0.8565	0.0020	0.0181
N	0.6389	0.4768	−0.0006	−0.0700	−0.30	0.7710	0.0051	0.0544
Plant Height								
X	41.2903	0.3271	0.1850	0.2609	1.01	0.3261	0.0567	0.0917
N	38.4211	1.4454	−0.5574	−0.4300	−2.16	0.0451	0.2158	0.3289
No. Branches								
X	−9.5090	0.7483	−0.0218	−0.1023	−0.45	0.6582	0.0118	0.2961
N	3.3925	0.0042	−0.0374	−0.2681	−1.15	0.2675	0.0718	0.0718

area. During a shadehouse study of interspecific competition between *A. elliptica* and the native *Psychotria nervosa*, a tortricid moth (Tortricidae) that is frequently found on *Psychotria* (A. Koop, personal observation) colonized both species heavily. As with *Psychotria*, the moth larvae rolled up terminal clusters of leaves and fed from within, removing an estimated 25–70% of the non-native's leaf area. Casual examination of leaf damage types in the field suggests that several other species of herbivores feed on leaves of *A. elliptica* and *A. escallonioides*. Finally, during a seed germination study in a disturbed area of Everglades National Park (Koop, 2004), both adult and larval bark beetles (Scolytidae) were observed within seeds of *A. elliptica*. Beetles were found at densities from one to about 15 within seeds and presumably were feeding on the seed's endosperm. Seeds preyed upon by beetles never germinated as they had over 75% of their endosperm eaten.

DISCUSSION—Consistent with predictions of the enemy release hypothesis (Keane and Crawley, 2002), the invasive non-native *A. elliptica* suffered less foliar damage than the native congener *A. escallonioides* and appeared to lack specialist above ground herbivores. The two species of folivores observed on *A. elliptica* were generalist herbivores that feed on several other local species (A. Koop, pers. observation). The bark beetle that fed on post-dispersal seeds of *A. elliptica* (Koop, 2004) may or may not be a specialized enemy. *A. escallonioides* however, does have a specialized moth (*Periploca* sp.) that parasitizes as much as 90% of developing seeds (Pascarella, 1996; 1998). Studies of other invasive species have reported damage by native herbivores on non-native species (Bowers et al., 1993; Schierenbeck et al., 1994; Creed and Sheldon, 1995). However, generalist and specialist herbivore communities on non-native species are generally not as diverse and abundant as on native hosts, and are likely to have reduced impacts (Strong et al., 1984; Keane and Crawley, 2002). This study casually surveyed for only above ground

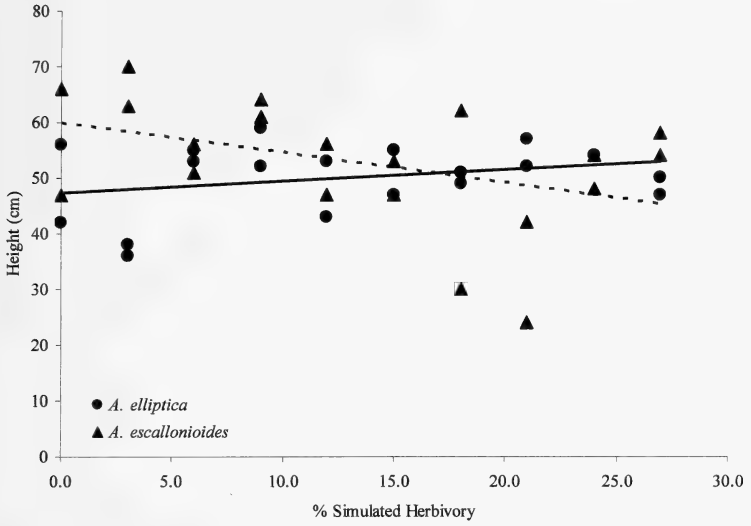

FIG. 3. Relative effect of increasing levels of simulated herbivory on final plant height (cm) on the invasive non-native *A. elliptica* (solid line) and the native *A. escallonioides* (dashed line). Standardized regression coefficients differed significantly between the two species ($t = 2.35$; $P < 0.05$).

herbivores. The impact of below ground herbivory, which can be very important in regulating some plants (Blossey and Hunt-Joshi, 2003) was not examined.

Results from the simulated herbivory experiment suggest that *A. elliptica* may have a high tolerance to herbivory relative to *A. escallonioides*. Over the one-year experimental period, levels of simulated herbivory of up to 27% did not reduce plant size or the number of branches of *A. elliptica*, while it did reduce the height of *A. escallonioides*. Only a few other studies have compared effects of herbivory between native and non-native species (Caldwell et al., 1981; Richards, 1984; Schierenbeck et al., 1994). In these studies, the non-native species displayed a compensatory response to herbivory by allocating more resources to stem and leaf production than the native. Why these native and non-native species would differ in their response to herbivory is not clear.

Response of final plant height of the native and non-native *Ardisia* species differed with increasing levels of simulated herbivory, suggesting that these species may differ in their overall response to herbivory. The non-native species responded positively to increasing levels of simulated herbivory, while the native species responded negatively. Differences in biomass allocation patterns between a native and non-native *Lonicera* sp. have been suggested to give the non-native species a substantial advantage over the native congener (Schierenbeck et al., 1994). Whether this is true for the invasive *A. elliptica* needs to be examined. Under natural conditions with limiting resources and interspecific competition, the effect of herbivory on plant growth and reproduction will likely be stronger (Erneberg, 1999, but see Thebaud et al., 1996) and negative for both species.

A simulation of population dynamics of *A. elliptica* using matrix models indicated that simultaneous reductions of over 58% in survival, germination and

reproduction are needed for population decline (Koop, 2003). If only a subset of life history processes were targeted, then reductions of 88% to 99% would be needed (Koop, 2003). Thus, if biological control were used as a form of management for *A. elliptica*, damage by either a single biocontrol agent or a set of agents targeting different host organs, must reduce life history transitions by at least 58%. Levels of up to 27% leaf area loss in this study did not have a significant impact on plant performance of the non-native *A. elliptica* over the one year experimental period. Even when damage by biological control agents is high (Lonsdale et al., 1995; Radford et al., 2001), it does not always have an equally similar effect on plant population dynamics because some targeted plant stages are not very important to population dynamics and growth (Lonsdale et al., 1995; Caswell, 2001; Koop, 2003). Longer-term studies that maintain herbivore damage for more than a year are needed to determine precisely what levels of herbivory are required to negatively impact plant performance and reduce growth and fertility transition rates (*sensu* Caswell, 2001) of the invasive, non-native *A. elliptica*.

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DISTRIBUTION AND ECOLOGY OF THE INTRODUCED AFRICAN RAINBOW LIZARD, *AGAMA AGAMA AFRICANA* (SAURIA: AGAMIDAE), IN FLORIDA

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ABSTRACT: *We document populations of the introduced African rainbow lizard (*Agama agama africana*) in Homestead, Miami-Dade County; Hollywood, Broward County; Palm City, Martin County; Punta Gorda, Charlotte County; and Sanford, Seminole County. The Homestead and Punta Gorda populations have been established for over 10 yr and have expanded at least 0.5 km from the point of introduction. The Palm City population has been established since 1999 and the Sanford population since 2000. All agamas were observed in urban or suburban situations perched on walls, rooftops, bridges, rocks, sidewalks, curbstones, or trees. We collected 33 voucher specimens from five populations 28 March 2002–11 March 2004. Maximum clutch size and maximum snout-vent length (SVL) of male and female* A. a. africana *in Florida exceeded those in native Nigerian populations. All adult females (≥94 mm SVL) collected May–August contained 5–18 vitellogenic follicles or oviductal eggs, but a female collected on 19 September was not gravid. Monitoring should be conducted to determine whether the species might eventually invade natural habitats and its potential impacts on native wildlife species.*

Key Words: *Agama agama africana*, African rainbow lizard, distribution, ecology, reproduction, introduced species, Florida

THE SUBTROPICAL climate of southern Florida has allowed the establishment and survival of many non-indigenous reptile species, particularly lizards of tropical origin. Florida presently contains the largest number of non-native amphibian and reptile species in the United States, but the list of 36 introduced species provided by Butterfield and co-workers (1997) did not include *Agama agama*, which has established populations in at least five counties in Florida. Bartlett and Bartlett (1999) reported *A. agama* as occurring near reptile dealerships in two suburban areas in Broward County and one area in Miami-Dade County, and they expressed uncertainty as to whether the species would "spread farther or succumb to climatic conditions that are very different from its native habitat."

Wilson and Porras (1983) noted a colony of *A. agama* (Linnaeus 1758) established since 1976 in the vicinity of NW 70th Avenue and 70th Street, Miami, Miami-Dade County. Voucher specimens of *Agama a. africana* Hallowell 1844 were collected from this Miami colony (LSUMZ 36647, UF 43490), but Wilson and

Porras (1983) did not include *A. agama* in their list of introduced herpetofauna because the population was apparently extirpated when the area was demolished to construct a rapid transit system.

In this paper, we describe the distribution, habitat use, and source of introduction of two large, well-established populations of *A. a. africana* in Homestead, Miami-Dade County, and Punta Gorda, Charlotte County, and three smaller populations in Sanford, Seminole County; Palm City, Martin County; and Hollywood, Broward County. Krysko and co-workers (2004) have already documented populations in Homestead, Punta Gorda, and Sanford, but we provide reproductive data for these populations.

In the early 1990s, agamas were either released or escaped during Hurricane Andrew at the residence of a reptile dealer near the junction of Coconut Palm Drive (SW 248th Street) and SW 163rd Avenue in the Redland area of Homestead (25°32.17′N, 80°27.13′W). The same reptile dealer responsible for the Homestead population subsequently relocated to 6362 Citrus Boulevard SW, which is ca. 11.7 km SW of Palm City (27°03.84′N, 80°19.28′W), where ca. 20 *A. a. africana* escaped or were released in 1999 and became established (Powell, 2003). An agama population also occurs in the vicinity of a reptile dealership on the south side of Stirling Road (State Road 848), east of its junction with NW 65th Avenue in Hollywood (26°02.76′N, 80°13.15′W). Approximately 17 years ago, a reptile dealer in Punta Gorda released *A. a. africana* at his private residence at 722 Solana Loop East (26°56.48′N, 82°01.81′W) (Clark, 2003), which is on the north side of State Road 17 (East Marion Avenue) ca. 1.3 km west of Interstate 75. The most northerly known agama population occurs in Sanford (28°46.18′N, 81°16.84′W). This population was introduced in 2000 when ca. 40 agamas escaped or were released from a nearby reptile store, which has since relocated (Ward, 2003).

Agama agama is often referred to as the African red-headed agama or common agama in the United States pet trade and popular literature (Frank and Ramus, 1995; Bartlett and Bartlett, 1999), and in scientific literature as the African rainbow lizard (Romer, 1953; Daniel, 1960; Chapman and Chapman, 1964; Harris, 1964; James and Porter, 1979; Cloudsley-Thompson, 1981; Sodeinde and Kuku, 1989). *Agama agama* is found in tropical, sub-Saharan Africa from Senegal east to Ethiopia and south to northern Angola and southern Tanzania. The coloration and pattern of this species varies over its geographic range, and nine subspecies are currently recognized (EMBL Reptile Database, 2003). The subspecies in Florida is apparently *A. a. africana*, which is imported for the pet trade from Ghana, Togo, and possibly Benin (Foster, 2003). Dominant, reproductive males in the five Florida populations we examined have tri-colored tails as described for this subspecies by Harris (1964) and are identical to photographs by James and Porter (1979) and Cloudsley-Thompson (1981) of *A. agama* from Ghana and Nigeria in West Africa. The reproductive coloration of adult males of *A. a. africana* consists of an orange head, indigo blue or black body and limbs, and a tail that is bluish white at the base and has an orange middle segment and black tail tip (Harris, 1964).

Agama agama is a sit-and-wait predator, feeding mostly on ants, orthopterans, and beetles (Harris, 1964; James and Porter, 1979) but occasionally on vegetation

during the dry season (Chapman and Chapman, 1964; Cloudsley-Thompson, 1981). In Africa, a few instances have been reported of *A. agama* preying upon their own young, small snakes, birds, and mammals (Harris, 1964; Cloudsley-Thompson, 1981).

METHODS—We visited all known sites for *A. agama* in Florida and checked out reported sightings, recording the presence of *Agama* and other introduced lizard species. One to five visits were made between 28 March 2002 and 11 March 2004 to Sanford, Punta Gorda, Palm City, Hollywood, Miami, Homestead, and Key Largo. Agamas were collected by hand, by using a blowgun that shot tapered corks, or by fishing using crickets for bait (Krysko, 2000). Voucher specimens and photographs were deposited in the Florida Museum of Natural History (FLMNH), University of Florida (UF collection). We reviewed the literature and obtained additional specimens from Everglades National Park (EVER) and the Louisiana State University Museum of Natural Science (LSUMZ) to corroborate identification.

We dissected all adult females collected to determine the number of oviductal eggs or follicles present. A female was considered ovigerous if the mean length of her elliptical oviductal eggs was $\geq$ 12.0 mm, and she was considered fecund if developing follicles were $>$ 3.5 mm in diameter (Daniel, 1960).

RESULTS—We observed agamas at seven sites in Florida but consider them to be established in only five areas: Sanford, Punta Gorda, Palm City, Hollywood, and Homestead. On 26 May 2003 in Sanford, we observed 13 *A. a. africana* on or around the large, vacant SunTrust Bank building at 3000 South Orlando Drive, five individuals farther north on the abandoned Gino's Café, and one individual perched on a boulder at the intervening car dealership. We collected six adult females (UF 136983-8). Most individuals were observed on brick buildings up to three stories tall, and the population was apparently very localized, although the surrounding habitat appeared suitable for colonization. The only other exotic lizard species observed was the brown anole (*Anolis sagrei*).

At three locations in Punta Gorda, we observed 22 agamas in rainy weather on 3 June 2003. At 1410 hr, three adult females were observed and one was collected (UF 137017) on the wall of a Circle K store (26°56.46′N, 82°01.89′W) and on a nearby oak (*Quercus* sp.) tree. At 1430 hr, 12 adults and one hatchling were observed on Solana Loop West (26°56.56′N, 82°01.86′W). At 1500 hr, two adult males and four adult females were observed on Solana Loop East at the original introduction site. On 4 June 2003, at least 23 adults and two hatchlings were observed at 1450 hr under the State Road 17 overpass of Lavilla Road (26°56.37′N, 82°01.64′W). One adult male, nine adult females, and two hatchlings were collected here (UF 137043–55). On 3 July 2003, four adults were observed at 1630 hr under the State Road 17 overpass of Florida Street (26°56.30′N, 82°01.50′W), ca. 0.6 km SE of the introduction site. Most agamas in Punta Gorda were observed on concrete embankments or the infrastructure of bridges, walls and roofs of buildings, or trees. Other exotic lizard species observed were the brown anole and red-sided curly-tailed lizard (*Leiocephalus schreibersii*) (Krysko et al., 2004).

At the Palm City site, we observed an adult female, collected two juveniles (UF 137409–10), and observed approximately 13 other juveniles or hatchlings on limestone rocks on 22 August 2003. We did not arrive at this site until 1815 hr on an overcast afternoon, which probably accounted for the paucity of adults observed.

Other exotic lizard species observed or reported introduced here (Powell, 2003) were the large-headed anole (*Anolis cybotes*), Guyana collared lizard (*Tropidurus hispidus*) (UF 137411–3), and spiny-tailed iguana (*Ctenosaura* sp.).

At the Hollywood site, we captured one adult male (UF 137674) and observed three females on property adjacent to a reptile dealership at 1430 hr on 22 August 2003. Other exotic lizard species observed around the reptile dealership were the brown anole, knight anole (*Anolis equestris*), brown basilisk (*Basiliscus vittatus*), common house gecko (*Hemidactylus frenatus*) (UF 137408), and flying gecko (*Ptychozoon lionotum*) (UF 137744).

We did not attempt to determine the exact geographic limits of the *A. a. africana* population in Homestead, but they were readily observed perched on sidewalks, low oolitic limestone and brick walls, and trees in front of the Redland Middle School at the junction of Coconut Palm Drive and SW 162nd Avenue and around houses and tropical plant nurseries up to at least three blocks from the school. We observed 25 individuals and collected one adult male (UF 131521) at the school and across the street on 28 March 2002. On 29 May 2002, we observed five adults and two hatchlings, collecting four adults (UF 132696–700). On 19 September 2002, we observed 20 individuals and collected one adult female (UF 134222). At 0930 hr on 22 August 2003, we collected two adult females (UF 137662–3) and observed two other adult females, one adult male, and ca. 20 juveniles or hatchlings during and after a rain shower. At 1300 hr on 11 March 2004, we observed three dominant reproductive males and 12 other adults at the school; the two individuals collected (UF 141218–9) resembled females but proved to be males upon dissection. Other exotic lizard species observed in the area were the brown anole and Amerafrican house gecko (*Hemidactylus mabouia*).

Individual agamas have been observed in the wild in at least two other counties in Florida, but we do not believe these sightings indicate reproducing populations. On 22 August 2003, we photographed an adult male *A. a. africana* (UF 141422) on the property of a reptile dealer at 16225 SW 172nd Ave., Miami. Other exotic lizard species observed were the giant ameiva (*Ameiva ameiva*) (UF 137671), common house gecko, golden gecko (*Gekko ulikovskii*), bark anole (*Anolis distichus*), brown anole, green iguana (*Iguana iguana*), and Nile monitor (*Varanus niloticus*). A male *A. a. africana* was photographed (UF 137389) in July 2003 in Buttonwood Bay, a subdivision on Key Largo, Monroe County (25°03.94′N, 80°28.46′W). This male and smaller, brown lizards were frequently observed (Kriss, 2003). During a visit on 21 August 2003, we did not observe the male, and the smaller lizards were not female agamas but instead northern curly-tailed lizards (*Leiocephalus carinatus armouri*) (Krysko et al., 2004).

All adult females collected 26 May 2002–26 August 2003 from Sanford, Punta Gorda, and Homestead contained either vitellogenic follicles or oviductal eggs, but a nongravid female was collected on 19 September 2002 in Homestead. A gravid female collected in Punta Gorda on 15 February 2004 oviposited eight infertile eggs on 9 March (Eddington, 2004). The mean length of oviductal eggs from eleven females ranged from 12.1 to 19.3 mm, and the mean diameter of follicles from 10 females ranged from 4.0 to 11.2 mm. The mean number of oviductal eggs was 9.0 ±

1.6 (n = 11), and the mean number of follicles was 9.7 ± 3.3 (n = 11). Clutch size (oviductal eggs or follicles) ranged from five to 18 and was positively correlated with snout-vent length (SVL) ($r^2 = 0.22$, $F_{1,20} = 5.67$, $P = 0.03$). Of 10 females collected in Punta Gorda on 3–4 June 2003, seven contained oviductal eggs, and the remainder contained developing follicles. Oviductal eggs probably represented second clutches, whereas follicles probably represented either second or third clutches. Two hatchlings captured in Punta Gorda on 4 June 2003 (UF 137053–4) measured 42 mm SVL and probably hatched from eggs laid > 60 days earlier. Overall, we captured 23 adult females ranging in size from 94 to 123 mm SVL (mean = 111 mm), four dominant reproductive males (122–154 mm SVL), and two males in nonbreeding coloration (92 and 105 mm SVL).

Discussion—We observed agamas perched on walls, rooftops, concrete curbs, bridges, rocks, and trees, and occasionally on the ground underneath shrubbery, on lawns, on sidewalks, or in parking lots. We never observed agamas in natural habitats. Even in Africa, *A. a. africana* is seldom observed in undisturbed habitats; instead, it is primarily found in close association with humans and is often the most frequently seen reptile species in urban and suburban situations (Romer, 1953; Daniel, 1960; Harris, 1964; Grandison, 1968; James and Porter, 1979; Cloudsley-Thompson, 1981; Sodeinde and Kuku, 1989). *Agama a. africana* is a tree-dwelling species that primarily inhabits savannas, but it has expanded its range into shrubland and forest areas that have been cleared for farms, villages, lawns, roads, trails, or buildings (Daniel, 1960; James and Porter, 1979). In rainforest areas, *A. a. africana* is restricted to living on the walls of houses (Harris, 1964; Cloudsley-Thompson, 1981). Manmade structures and debris piles in disturbed habitats are preferred perching and roosting sites (Grandison, 1968). Agamas are extremely fast, and when approached, quickly seek shelter in cracks on walls, on trees or shrubs, beneath debris, or in or under concrete structures (Cloudsley-Thompson, 1981).

In Africa, *A. a. africana* is especially active on hot sunny days and attempts to avoid wind and rain (Cloudsley-Thompson, 1981). In Florida, we sometimes observed agamas during rainy weather and immediately after rain showers, with juveniles and females appearing most tolerant of wet conditions. The more northerly agama populations in Florida are probably reliant on warm refugia to escape occasional freezing temperatures. The air temperature in Sanford on 24 January 2003 reached –2.8°C. We observed agamas in Sanford entering holes in the walls of abandoned buildings and stormwater grates that led to buried pipes. We suspect that this population is able to survive low winter temperatures by accessing the interiors of buildings or underground refugia.

In Africa, *A. a. africana* oviposits multiple clutches consisting of 3–9 eggs each, with five or six eggs being most common (Daniel, 1960; Harris, 1964). In comparison, females from Florida populations contained 5–18 (mean = 9) oviductal eggs or vitellogenic follicles. We suspect agamas in Florida oviposit at least three clutches annually, giving a minimum annual reproductive output of approximately 27 eggs. In Nigeria, females become sexually mature at ca. 90 mm SVL and ca. 14

months of age (Harris, 1964; Sodeinde and Kuku, 1989). In Liberia, both sexes attain sexual maturity at ca. 80 mm SVL and their second year of life (Daniel, 1960). Our smallest gravid female (94 mm SVL) contained five follicles.

Considering the collection dates of gravid females and hatchlings, the size of follicles in dissected females, and assuming an incubation period of ca. 58 days (Sodeinde and Kuku, 1989), the breeding season in Florida probably begins in February. African hatchlings measured 30–38 mm SVL (Romer, 1953; Daniel, 1960; Harris, 1964), indicating that small individuals (42 mm SVL) collected on 4 June 2003 were probably not recent hatchlings. In its native range, *A. a. africana* breeds year-round in the rainforest belt and Cape Coast, Ghana (Daniel, 1960; James and Porter, 1979), but in drier savanna regions, the breeding season coincides with the rainy season (Harris, 1964). Cool weather probably restricts the breeding season in Florida, particularly for the more northerly populations. Females still contained well-developed eggs in late August, but a female collected in 19 September was not gravid, suggesting that the breeding season ends in late summer in Florida.

We observed relatively few hatchlings in May and June, as also noted by Bartlett and Bartlett (1999). For the first two months of life, hatchlings avoid adults and spend most of their time in dense vegetation, often near the ground (Harris, 1964). By four months of age, juveniles typically live gregariously within a particular territorial group (Harris, 1964). During visits in August to the Homestead and Palm City populations, however, we observed numerous hatchlings and juveniles using the same perches as adults, and hatchlings and juveniles were also observed in August in the Sanford population (Ward, 2003). In Homestead, we saw hatchlings most frequently where the oolitic limestone wall was broken resulting in numerous interstices in the jumble of rock fragments. These small spaces presumably provided hatchlings with shelter from adults or other potential predators.

Our largest female (123 mm SVL; UF 137043) and male (154 mm SVL; UF 137052) exceeded the maximum size found for a female (119 mm SVL) and male (148 mm SVL) in two Nigerian populations (Sodeinde and Kuku, 1989). In Nigeria, the mean SVL of adult females was 97 mm ($n = 68$) in one population (Harris, 1964) and 104 mm ($n = 28$) in another population (Sodeinde and Kuku, 1989), compared with 111 mm SVL ($n = 23$) in Florida. In Nigeria, adult males averaged 128 mm SVL ($n = 50$) (Harris, 1964) or 125 mm SVL ($n = 40$), which corresponds to ca. 22 months of age (Sodeinde and Kuku, 1989).

Presently, populations of *A. a. africana* are established in at least five counties in Florida, and two of the populations have persisted for at least 10 years and dispersed at least 0.5 km from their point of introduction. Additional undocumented populations are probably present. We learned that most *A. a. africana* populations in Florida resulted from many individuals escaping from reptile dealers (Hurricane Andrew might have been responsible for the Homestead population) or being intentionally released. Reptile dealers or hobbyists sometimes release lizards in attempts to establish feral populations for future exploitation (Krysko et al., 2003), but we suspect that this is not the primary source of introduced *A. a. africana* in Florida. Imported *A. a. africana* are readily available and inexpensive, and capturing feral individuals without damaging them would be difficult and uneconomical,

unless accessible nocturnal refugia could be located. *Agama a. africana* are typically imported for $0.75 and wholesale for $1.50–$3.00 (Powell, 2003). Specimens tend to do poorly in captivity, often failing to settle down or feed well (Harris, 1964). Some agamas are probably released because they are nonsalable due to low market demand or poor physical condition, whereas others are released because persons desire seeing them around their residences. Conversations with local residents of neighborhoods containing agamas indicate that they enjoy observing them and do not want to see them captured. The general public is usually unaware that it is illegal to release non-indigenous animal species in Florida.

Some confusion exists as to the identity of all specimens of agamas observed in Florida. Bartlett and Bartlett (1999) photographed a dominant male that was one of many individuals observed on the walls of a reptile dealership in Hollywood, Broward County (Bartlett, 2003). During a visit there in August 2003, we observed four adult agamas, and the adult male collected was *A. a. africana* and did not resemble the aforementioned photograph, which was possibly of an East African subspecies. According to the reptile dealer, large numbers of agamas are not currently present, and earlier observations may have been after many had recently escaped. In 1996, a lizard (EVER 304176) identified as the common spiny agama (*A. hispida*) was collected at the corner of Coconut Palm Drive and SW 167th Avenue, the introduction site for the *A. agama* population in Homestead. We examined this specimen and identified it as the hardun or starred agama (*Laudakia* [*Agama*] *stellio*), which is native to Greece, southwestern Asia, and northern Egypt (Arnold, 2002). *Laudakia stellio* has occasionally been collected in Miami-Dade County but is apparently not established (Meshaka et al., 2004). Another *L. stellio* that was misidentified as *A. hispidus* (EVER 308085) was collected in 1999 ca. 6.5 km SSW of the Redland Middle School.

More populations of *A. a. africana* may become established in the southern half of peninsular Florida. The agamas we observed were restricted to open, human-altered environments, where they tended to use vertical surfaces, such as walls or bridges, containing crevices or holes. Although agamas presently occur only in suburban or urban situations, we suspect this species could survive in open, agricultural areas with suitable perch sites and refugia. Like many other exotic lizard species in Florida, *A. a. africana* may be unable to successfully establish populations in undisturbed natural habitats. Impacts of agamas on native wildlife species in Florida are unknown.

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